Characterization of psychological and biological factors in an animal model of Warrior Stress

by

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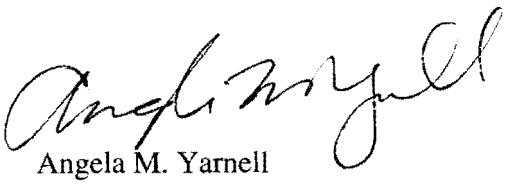

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ABSTRACT

Title of Dissertation: Characterization of psychological and biological factors in an animal model of Warrior Stress

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For over a decade, American service members have been vigorously defending this nation and, in the process, have been exposed to death or the threat of death, explosive blasts, debilitating injuries, and other environmental stressors (*e.g.,* noise, heat), not to mention separation from loved ones and unpredictable deployment schedules. Because service members are exposed to physical and psychological stressors, it is important to understand the effects of stress on psychobiological processes to better prevent and/or treat resulting illness or injury. Despite increased awareness that Warriors exposed to stress and blast may experience cognitive effects (*e.g.,* memory and attention problems) and other post-deployment symptoms (*e.g.,* chronic pain) mechanisms underlying psychological effects of stress and blast injury have yet to be identified. Therefore, basic research must be conducted to understand the complex response to injury and stress.

The purpose of this doctoral dissertation project was to characterize psychological and biological responses in a rodent model of Warrior Stress using males and female rats.

A comprehensive and sophisticated multivariate data analytic strategy examined complex relationships among behavioral and biological responses to physical and psychological stress. Data were gathered from an experimental investigation of blast overpressure induced traumatic brain injury and psychological stress in male and female rats (N=96). Exploratory factor analysis, multivariate analysis of variance, and multiple discriminant analysis were performed to: (1) reveal underlying dimensions in the data; (2) use those dimensions to try to identify the mechanisms of differential effects of four levels of stress; and (3) determine whether there were sex differences for psychobiological variables.

Multivariate analyses revealed that **males and females differed on dimensions that represented neurochemical function** of: (1) serotonin among the prefrontal cortex, hippocampus, insula cortex, and basolateral amygdala, and (2) dopamine activity among the prefrontal cortex and the amygdala. There were no apparent differences among stress groups on combinations of the psychobiological variables. This project highlights the need for considering sex differences in psychobiological responses and for including males *and* female participants in all injury and stress research.

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The longest period of war in U.S. history is coming to an end. However, the war will "continue" in the physical and psychological lives of Wounded Warriors. For over a decade, American service members have been vigorously defending this nation and, in the process, have been exposed to death or the threat of death, explosive blasts, debilitating injuries, and other environmental stressors (*e.g.,* noise, heat), not to mention separation from loved ones and unpredictable deployment schedules. Despite the wars ending, effects of these stressful exposures will persist in the lives of American service members. Therefore, it is important to understand physical and psychological effects of war experiences and particularly injuries or variables (*e.g.,* stress) that could have short and long-term health effects. This doctoral research project used data gathered from a recent experimental investigation using a preclinical animal model of Warrior Stress to characterize complex relationships among physiological (peripheral and central) and psychological responses. The particular multivariate statistical analyses conducted were based on review and consideration of the extant stress research literature and were designed to reveal patterns and associations among psychological and biological variables to inform prevention and treatment of deleterious effects of stress experienced by Warriors.

Traumatic Brain Injury

Traumatic brain injury (TBI) is a physical stressor experienced by Warriors. During the last 12 years, 262,065 cases of TBI were reported to the Department of Defense, with nearly 77% meeting criteria for mild TBI (73). Reports indicate the majority of these TBIs are closed head injuries, and more than half result from exposure to blast (117; 157; 170; 255). This doctoral dissertation work used data gathered from an

experiment in which rats were exposed to a blast overpressure wave, a method of injury used to model exposure to improvised explosive devices (IEDs; 148).

TBI has been associated with cognitive impairment, including deficits in complex attention, executive function, and memory (25; 26; 34; 141; 176; 209; 242); as well as headache (251); arm, leg, and joint pain (251); and chronic pain (76; 112). In a majority of mild TBI or concussion cases, full recovery occurs and no persistent cognitive or other symptoms persist (119), but some individuals experience effects of mTBI or concussion that last for weeks, months, or longer, referred to as post-concussive syndrome (32; 195). Post-concussive syndrome is a constellation of symptoms that may present following TBI which include: headache, dizziness, irritability, problems with attention and memory that persist beyond the period of time typical for healing from the TBI (10). Many clinicians and researchers have begun to question whether or not post-concussive symptoms can be completely attributed to brain injury in military and other populations. Other factors, like stress and trauma exposure, and disorders, like post-traumatic stress disorder (PTSD) and depression, have been associated with post-concussive symptoms and problems following brain injury (27; 117; 251). Because of these associations and because Warriors are exposed to physical and psychological stress in addition to TBIs, it is important to consider how stress may be contributing to Warriors' "post-deployment" problems.

Warrior Stress

Stress is an occupational hazard of military service and Warriors are exposed to all types of physical (*e.g.,* noise, sleep deprivation, extreme temperatures) and psychological (*e.g.,* threat of attack, separation from family) stressors. These stressors are associated with increased risk of physical and mental health consequences which

disrupt day-to-day life as well as performance under pressure (243). These disruptions manifest in the following complications: sensory and motor problems, problems with sustained attention and learning, anxiety, depression, and PTSD. According to the 2011 Joint-Mental Health Advisory Team (125) which surveyed approximately 1,000 deployed Soldiers and Marines, in 2011 there was more combat exposure, greater rates of anxiety, depression, and acute stress disorder than rates reported from 2003-2010. Various other studies of Veterans report marked percentages meet criteria for major depression, PTSD, and alcohol misuse after deployment (e.g., 116). Post Deployment Health Assessment (PDHA) data from all Army Soldiers and Marines between the years of 2003-2004, revealed 35% of Veterans who served in Iraq sought mental health services in the year following their return and 12% of these troops were diagnosed with a mental health problem (115). Additionally, multiple deployments have been associated with more psychological problems, decreased morale, and increased use of medications to treat mental health problems, combat stress, and sleep problems (125).

Stress, TBI, and PTSD

The similarities in presentation of mTBI and PTSD have been the subject of much debate and research for the last few years. One hypothesis is TBI, especially with a loss of consciousness, places Warriors at increased risk for development of PTSD, depression, and other "post-deployment symptoms" because these disorders are strongly associated with dysregulation of autonomic and neuroendocrine systems (251). This dysregulation may occur in response to stress or as a result of injury. Additionally, brain areas involved in cognitive and emotional regulation, such as the hippocampus, white matter tracts, frontal areas, amygdala, pituitary (31; 129), are vulnerable to TBI and may also be

associated with PTSD (129; 165; 244). This doctoral project investigated the relationship between physical and psychological stressors and psychobiological outcomes within a data set that included behavioral indices of psychological functioning, levels of stress hormones (corticosterone, adrenocorticotropic hormone [ACTH], prolactin) measured in the periphery, and activity in relevant brain regions (prefrontal cortex, amygdala, hippocampus, insula cortex) from rats exposed to blast, psychological stress, and a combination of blast and psychological stress.

Because service members are exposed to physical and psychological stressors, it is important to understand the effects of stress on psychobiological processes to better prevent and/or treat resulting illness or injury. Despite increased awareness that Warriors exposed to stress and blast may experience cognitive effects (*e.g.,* memory and attention problems) and other post-deployment symptoms (*e.g.,* chronic pain), the available Warrior and patient information does not establish causal relationships or identify mechanisms underlying psychological effects of stress and blast injury. Therefore, basic research must be conducted to understand the complex response to injury and stress. *This doctoral project used data from an experimental investigation of blast exposure and psychological stress in rats that allowed for causal inferences to be made.*

Stress is a complex process that includes psychological and physiological responses. Stress research up through the 20th Century focused on **psychological and physiological systems** operating **independently** in response to **single stressors** with biological measurements mostly in the **periphery**. It is important to determine whether and how psychological and physiological systems **interact** in response to **simultaneous exposure to more than one** type of stress considering **central physiological** changes,

such as neurotransmitters, which may influence psychological function. Because many service members are subject to psychological stress and physical stress, such as brain injury, there is a need to examine the effect of multiple stressors acting simultaneously. *The purpose of this doctoral work was to gain a better understanding of the complex response to multiple stressors which may help to improve treatment as well as prevent comorbid stress and injury conditions such as PTSD.*

Purpose of Doctoral Project

This work used data gathered from a comprehensive experimental investigation of psychological and physiological effects of blast overpressure (BOP) induced traumatic brain injury (bTBI) and psychological stress in male and female rats to determine effects of blast, stress, and blast + stress on behavioral (measures of attention, memory, nociception) and physiological (peripheral stress hormones and central neurotransmitters) responses (see Study of bTBI and Stress section for additional details, p. 41ff.). The author participated as a co-investigator in the design and collection of all data used in this doctoral project. A small portion of the data have been analyzed separately for scientific meetings (22; 259) and for the author's Master's project (257). This project, in contrast, examined the data from a multivariate approach and used data that have not been thoroughly analyzed to date to determine the relationships among psychological and biological outcomes.

Advanced data analytic techniques were used in this project to examine specific hypotheses regarding the complex relationships among the behavioral and biological variables. The aims of this project were to examine psychobiological variables gathered from an experimental investigation of Warrior stress: (1) to identify strongly related

variables and to use these related variables to build multivariate models, and (2) to determine how different levels of stress account for relationships among these variables in male and female rats.

This doctoral dissertation first provides background information about the stress process, relevant biology, and the psychobiology of stress, with an emphasis on the effects of stress relevant to the present project (*i.e.,* on memory, attention/arousal, and pain perception). In addition, this background provides a description of the experimental design and methods of the project from which the data were collected. Next, a detailed description of the statistical analytic approach and methods used in the present project are presented. The results, discussion, summary, and conclusion follow.

Background About the Stress Process

Definition

The following description and definition of stress to be used in this proposal was constructed based on various sources (e.g., 19; 104; 138; 163; 170; 217). Stress is a complex process that begins with the perception of a threat or challenge in the form of physical or psychological stimuli. If the stimuli are perceived to demand physical and psychological resources, then there is an integrated physiological and psychological response by the organism. The purpose of this response is to preserve life and physical or psychological well-being. Whether or not the stimuli are perceived to be a threat depends heavily on individual characteristics which include but are not limited to: genetics, personal experience, coping mechanisms, and available social support. If the physiological and/or psychological response is compromised in some way (*e.g.,* overactive or underactive), then physical or psychological injury or disease may occur. Responses to acute stressors usually are adaptive, whereas responses to repeated, chronic, or extreme stress often are disruptive to health.

Historical Perspective

Historical conceptualizations and definitions of stress framed the field of study and help to shape the current understanding of the complex systems involved, but each was limited by the knowledge and techniques of the time.

Adaptation. The earliest conceptualizations of stress were heavily influenced by Charles Darwin's work (70), specifically organisms that can adapt to their environments survive to pass on their genes, and those that do not or cannot die off. The concept of adapting to an environment was picked up by the French physiologist, Claude Bernard.

Bernard is known for his concept, *milieu intérieur,* or internal environment, which states an organism's ability to move freely in the external environment depends on the capacity of its internal environment to respond to threats from the external environment (30). For example, a lizard can move around freely, but its body depends on the environment for warming and cooling. A mammal, on the other hand, has bodily systems that will adjust to the environment around it for heating and cooling. The influence of Darwin is evident in Bernard's work. An organism that can adapt to its environment is more likely to survive. Cannon, who characterized the ability of specific bodily systems to adapt, was influenced by the work of Darwin and Bernard.

Homeostasis. Walter Cannon, M.D., was an American physiologist who used the term homeostasis to describe the body's natural tendency to maintain balance and stability in essential biological systems in the midst of the challenges of change (49). Cannon extended Bernard's work by observing how the body responded to challenges with a series of chaotic, disconnected responses, for example: there was release of adrenalin (epinephrine) from the adrenal medulla, a decrease in digestive activity, and an increase in blood flow to the heart, lungs, brain, and large muscles. As another intellectual follower of Darwin, Cannon reasoned that though the responses seem to be disorganized, they had to serve some adaptive function. His characterization of the "fight or flight" response (48) reasoned that the sympathetic division (SNS) of the autonomic nervous system was activated by internal and external stimuli in the environment and these responses were adaptive and self-preserving. It is from this work of Cannon that the adaptive and life-preserving aspects of the stress response were first understood. Yet, Cannon also observed a point where homeostasis was no longer attainable, and the

body's ability to adapt in the face of change "broke down," resulting in injury, illness, and death. It is at this point where Selye's work on the General Adaptation Syndrome (GAS) became relevant.

Breakdown of adaptation. Hans Selye, M.D., a Hungarian endocrinologist, is credited with coining the term "stress," which he borrowed from the field of physics. He defined stress as the non-specific response of the body to any demand for change or adaptation (217). Selye published extensively on the subject of stress. Over his 50 years of research, he authored or co-authored more than 1,700 publications, including 40 books (235). Selye's contributions include an understanding about what constitutes a stressor, and the role of hormones in the stress response. During his early years in medical school, Selye observed that many diseases presented with similar symptoms: *e.g.,* loss of appetite, joint pain, and fever, and it was up to the physician to discern the subtle differences that made the specific diagnosis possible (84). This concept of specificity would lead him to discover that to some stimuli there is, in fact, a "non-specific" response (217). Selye defined the GAS as "the sum of all non-specific, systemic reactions of the body which ensue upon long continued exposure to stress (217, p. 119)." The GAS has three distinct stages: alarm reaction, resistance, and then exhaustion (217). The alarm stage represents a reaction to stimuli during which the organism has not yet adapted. Resistance occurs after repeated presentations of the same stimuli to which the organism adapts. When adaptation is no longer possible (because of a variety of different factors) or can no longer be maintained, then exhaustion occurs and future resistance is not possible. Selye believed that during the stress response, certain hormones were synthesized and released from the anterior pituitary in the brain and the adrenal cortex

(near the kidneys) in excessive amounts to increase the organism's resistance to the stressor (217). Despite his observations, Selye still had some doubt about mechanistic systems through which non-specific stressors and hormonal release were causing these effects. He maintained that a defensive endocrine response was valuable in its facilitation of adaptation, but this system was subject to malfunction with extended stimulation. He called this malfunction endogenous hormone over-dosages, which he thought caused certain cardiovascular, renal, and joint problems (218). From this notion he developed the concept that many of the most common maladies of man are "diseases of adaptation" or the by-products of abnormal adaptive reactions to stress (219).

Another way Selye helped to define a stressor is he discovered that even changes considered joyful elicit a response in the body. He differentiated the concepts of eustress ("positive" stress) and distress ("negative" stress) and demonstrated that both activate the same physiological mechanisms that help people respond to stress (219). Eustress occurs when we are required to adapt to an event or circumstance we perceive as positive (*e.g.,* new job, new relationship, return from deployment, marriage). Distress occurs when we are required to adapt to an event or circumstance we perceive as negative (*e.g.,* losing a job, unexpected/unwanted deployment, divorce, death of a loved one). Distress causes more damage to our bodies. Distress is the more commonly referenced type of "stress."

Psychological stress response. The early conceptualizations by Bernard, Cannon, and Selye were derived from the treatment of individual patients; they did not account for the role of psychological responses to stress. It was another physician, William Beaumont, who first considered the role of psychological variables in the stress process. Beaumont, a U.S. Army surgeon, was well known for his contributions to

digestive physiology. While treating abdominal wounds in the field, he observed the stomach lining and its secretions, and he discovered that extreme emotional states like anger or anxiety inhibited digestive activities (84). It was not until the mid-20th century that psychologists and psychobiologists picked up on Beaumont's work and helped to broaden understanding of stress to include psychological stressors.

John W. Mason, M.D., was one of the first stress researchers to conceptualize stress as an integration of the activity of multiple endocrine systems, cognitive variables, personality factors, and environment/situational variables (159; 160; 162). Though he is now thought of as a stress psychobiologist because of his consideration of the interaction between psychological, environmental, and biological variables, most of Mason's work was primarily conducted first as a physician with the Division of Neuropsychiatry, Walter Reed Army Institute of Research, and then later in the Department of Veteran Affairs. His studies stretched from animals in the laboratory to military populations in the field. Mason was a critic of Selye and disputed the "non-specific" response definition and the emphasis on physical stressors having the greatest impact on bodily systems (163). He argued psychological factors were necessary for the adrenal response to occur, and this psychoendocrine response was primarily anticipatory in nature (161; 163). With studies of combat pilots, Mason reported complex psychological defenses could be used to alter the way that threats were perceived and this alteration could actually minimize the perception of danger and lead to feelings of invincibility and invulnerability (35).

In addition, Mason understood that hormonal responses influenced each other. He called for individual hormones, released during and after stress, to be evaluated in a multi-hormonal context because he understood that individual hormones have multiple

and sometimes opposing effects, and they interact in a complex, mutually regulatory manner (159). He proposed multiple interacting hormonal systems were influenced by "cortical" (*i.e.,* pituitary) areas, but he did not have the advantage of modern technology to assess these systems simultaneously (159). His understanding of the interaction was the basic relationship between hormones triggering the synthesis and release of other hormones (*e.g.,* adrenocorticotropic hormone [ACTH] and cortisol) or how hormones are related to each other in the metabolic sense, such as the relationship between insulin and cortisol. He did not, however, conceptualize how multiple central and peripheral systems interacted. *This project examined the interaction of central and peripheral stress responses.*

Mason was convinced that the stress process includes psychological as well as physiological mechanisms (159). He believed what was historically known about effects of physical stressors was confounded with effects of psychological stress. Through a series of experiments and studies in the late 1960s, he attempted to demonstrate that when psychological components were reduced or eliminated, peripheral adrenocortical responses were also eliminated/reduced (159-161). *This work examined effects of a physical and a psychological stressor, each presented alone and in combination with each other.*

Mason assumed psychological factors influenced biological processes. He did not think psychological and biological processes interacted in a bi-directional manner. *This project allowed for an investigation into bi-directional interactions of physiological and psychological systems.* Mason's work influenced the work of Richard Lazarus and Susan Folkman and their contributions on the role of appraisal and coping in the stress process.

Appraisal. Lazarus and Folkman made theoretical and empirical contributions and broadened the understanding of stress to include cognitive and emotional factors in the stress process. They defined stress as a relationship between the person and the environment that is appraised by the person as taxing or as exceeding his/her resources and endangering his/her well-being (138). The influence of Bernard and Cannon on the role of the environment in the stress process, as well as the influence of Mason understanding that psychological factors are automatic in the perception of stress, can be seen in Lazarus and Folkman's definition. The cognitive process of appraisal and the idea that a threat can be down-graded based on a person's experiences is exactly what Mason was describing in the study of combat pilots (35). Lazarus and Folkman's contributions also include the idea that, in addition to the stress response, a person possesses coping mechanisms which also play a role in the perception of and reaction to stress (138). Despite their extensive contributions, Lazarus and Folkman's model does not include consideration of any biological stress responses or central processes underlying the psychological components. Similar to Mason, their model lacks consideration of any bi-directional effect of psychological and biological processes. The work of these psychologists and this psychobiologist (*i.e.,* Mason) helped to broaden understanding of stress to include psychological stressors, but did not focus on the role of the psychological response to stress. If the work did acknowledge psychological responses, then it stopped short of attempting to identify the underlying or associated physiological responses or systems. *The aims of this project were to examine physiological responses, physiological systems underlying psychological concepts (e.g., memory), and behavioral responses to two different stressors.*

Modern Perspective

Adaptive and non-adaptive responses. Stress can have adaptive (beneficial) and non-adaptive (harmful) effects which depend on timing, duration, intensity, and individual differences. The acute psychological and physiological effects of stress usually are adaptive and beneficial. The activation of the sympathetic nervous system mobilizes the bodily systems to meet the demands placed on them. The activation of psychological processes, such as cognitive appraisal, allow the person to call on past experience and survey available resources to determine how to handle the threat or challenge with which (s)he is faced. The simultaneous activation of these systems is essential to preserve physical and psychological well-being. When these systems are disrupted by repeated, chronic, or traumatic stressors or physical disease or injury, then the adaptive processes break down (169; 217). Additionally, these systems may not just cease to work but, instead, may become overactive and result in even more insult or injury to physical and psychological well-being.

Allostasis and allostatic load. The concept of allostasis builds on the work of Cannon and Selye and was presented by Sterling and Eyer as an active process by which the body responds to daily events and maintains homeostasis -- a concept of attaining stability through change Sterling and Eyer (230). Allostasis can be carried out by a number of systems including alterations in: hypothalamic-pituitary-adrenal (HPA) axis hormones, the autonomic nervous system, and inflammatory markers (*e.g.,* cytokines), and is generally adaptive in the short term. Over time, however, homeostatic set points are shifted and reset as a result of repeated adaptations.

Bruce McEwen and colleagues extended the concept of allostasis to describe allostatic load/overload (170; 172). Allostatic load represents the cost of stress on the bodily systems and the conditions under which excessive stress or mis-management of allostasis (*e.g.,* response of systems in the absence of stressors, inadequate response to stressor, lack of appropriate adaptation after repeated activations) occurs (170). Like Selye, who conceptualized GAS as a breakdown in homeostatic mechanisms in response to stress, McEwen identified allostatic overload to be the mechanism by which stress contributes to physical disease and emotional/behavioral disorders (170; 172). Unlike Selye, McEwen focused on the role of the brain and central mechanisms in the stress process (170). He described the brain as both the target of stress and as the control center for the subsequent biological and behavioral responses (170). *This project examined data gathered from peripheral and central stress response systems.*

McEwen, like Mason, recognized the complexity of the stress process and the many interacting systems that are involved. He postulated that the body responds to stress with the release of "chemical mediators" which, in the acute stage, promote change in bodily systems to meet the demands of the stressor. For example, activation of the sympathetic nervous system (SNS) results in the release of catecholamines from the adrenal medulla to increase heart rate and blood pressure. This activation could occur when a Warrior sees an enemy with a rocket propelled grenade pointed at the convoy and facilitate the Warrior's quick action to neutralize the threat, or it may simply occur in response to a positional change of moving from the prone position on the ground to a standing position. The concept of allostatic load/overload describes the potential for pathophysiologic changes in the response with repeated activation or other malfunction

(e.g., as a result of injury; 172). In addition, in this hypothetical case, there may be increased activity in the prefrontal cortex (PFC) which, in turn, may inhibit emotional reactions in the amygdala. *This project's analysis of central and peripheral chemical activity along with behaviors addressed this type of possibility.*

McEwen went beyond Mason by hypothesizing that the relationship of interacting systems is nonlinear, systems can be mutually regulatory, and a change in any one chemical mediator results in a compensatory change in the other mediators involved in the process depending on other factors, such as time and intensity of the change in each participating mediator. In a review of the physiology and neurobiology of stress, McEwen (170) stated that the complexities of these interacting systems could not (then) be understood because biomedical methods available at that time were unable to measure the systems simultaneously. *This project attempted to characterize the relationship between multiple central and physiological systems in response to stress by taking each measurement from the same sample of subjects with identical exposures and use advanced, multivariate statistics to interpret the outcomes on physiological measures. In addition, this work included behavioral indices of memory, arousal, and pain.*

Individual differences. The perception and experience of stress is highly individualized. The early conceptualizations of stress presented by Cannon and Selye did not consider individual differences in the stress response. Mason, with a focus on the role of psychological variables, and Lazarus and Folkman's model, that accounts for past experiences, memories, biases, and childhood experiences that shape the appraisal and coping processes, expect that individuals will respond differently to stressors based on their personal history and experiences. Taylor, Klein, and colleagues (239; 240) also

theorized about how individual differences are relevant to the stress process; specifically that males and females respond differently to stress. Their conceptualization has come to be known as the "tend and befriend" response of females to stress and it stands in contrast to Cannon's "fight or flight" response (that primarily was based on studies of males). The postulated female stress response is based on the fact that females of any species may be pregnant or responsible for caring for their young and may not be able to fight an enemy or flee from a threat. Therefore, the female sex has adapted and has evolved to have an additional response system, mediated through other chemicals (*e.g.,* oxytocin) to deal with stressors. Taylor, Klein, and colleagues present an interesting notion that may account for differences in behaviors or responses for males and females that are exposed to stressors. For that reason and because there are male and female service members exposed to stress, *male and female responses were considered in the present work.*

Biological Stress Response

The classic biological mechanisms underlying stress responses are activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. SNS activation results in a series of cardiopulmonary and muscular changes that mobilize energy and support organs necessary for "fight or flight" (48) via catecholamine release in the central and peripheral nervous systems (20; 60). SNS activation is not directly relevant to the present work, so it is not discussed in this proposal. HPA axis activation is directly relevant to the present work because excessive activation of this system may result in damage to brain areas (*e.g.,* hippocampus, amygdala, prefrontal cortex) relevant to the psychological constructs of interest to this project; therefore, it is discussed in detail below.

The HPA axis and the relationship between corticotrophin releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol are important for the stress response. The paraventricular nucleus of the hypothalamus makes corticotrophin releasing hormone (CRH) and secretes it from the median eminence to the pituitary through a portal circulation system (109). CRH signals the synthesis and release of ACTH from the anterior pituitary. ACTH then travels through the bloodstream to its target tissue, the adrenal cortex. The presence of ACTH in the blood signals the synthesis and release of other hormones; especially important for stress is cortisol (in humans; corticosterone is the analogous biochemical in rats). Cortisol has many actions in the body and its goal is to mobilize energy to meet the demands of the stressor. Cortisol (a glucocorticoid) increases the level of glucose (sugar/energy) in the blood to be used by the brain, heart, muscles by metabolizing fat stores and inhibiting insulin (63). Cortisol also inhibits the immune system to reserve energy for other systems (63). *This project used measures of peripheral stress hormones related to HPA axis activation (corticosterone, ACTH, and prolactin) and central physiological processes gathered from rats exposed to psychological stress and blast in statistical analyses to determine if relationships existed among these stress response variables. In addition, behavioral indices of psychological constructs were used in the statistical analyses.*

The Papez circuit, first described by James Papez in the late 1930s and later modified by Paul D. MacLean, is a set of neural anatomical structures that form a pathway in the brain thought to underlie the formation of emotional memories. This set of structures, with the addition of a few others (*e.g.,* amygdala), is now commonly referred to as the limbic system (131). The areas of the brain involved in this circuit

include the hippocampal formation, fornix, mammillary bodies, mammillothalamic tract, anterior thalamic nucleus, cingulum, entorhinal cortex, linking back to the hippocampal formation (221). The continued examination of the functional role of this circuit, since its discovery, has revealed that it also plays a key role in memory formation (155). *Brain structures of the limbic system included in this dissertation project were the hippocampus, the basolateral, and central nuclei of the amygdala.*

Psychobiological Effects of Stress

This project, including the empirical investigation from which data were drawn as well as the multivariate statistical analyses conducted, focused on complex psychobiological relationships. More specifically, this project investigated: (1) stress and memory; (2) stress and attention/arousal; and (3) stress and pain. These three constructs were selected because they are common symptoms and functional problems that overlap in patients with TBI and PTSD (26; 116; 244). The following sections present information that forms the conceptual bases of the variables examined in the research. Emphasis is placed on biological variables underlying psychological constructs.

Stress and Memory

Stress has been associated with impairments in learning and memory (75). The hippocampus is the primary brain region associated with memory formation. The hippocampal structures are part of the cerebral cortex located in the medial temporal lobe (in primates) and composed of the entorhinal cortex, dentate gyrus, and *Cornu Ammonis* (CA) pyramidal neurons 1 - 4. Activation of CA 1 neurons results in signal amplification (via intracellular calcium cascades) which potentiates as it travels through the remaining CA neurons. This process is called long-term potentiation and is thought to be the mechanism by which memories are formed. Additionally, the hippocampus projects to the amygdala. This connection underlies the mechanism by which associations, or memories, become encoded with emotional valence or salience (60).

The synaptic adaptability of the cells in the hippocampus are thought to underlie the formation of memories; damage to this area, may explain the effects of stress on

memory. Because of its dynamic (versus static) properties, the hippocampus is susceptible to damage and over-excitation (109). TBI or physical damage to the brain is associated with memory impairment (178), and stress can cause remodeling/restructuring to the hippocampus and surrounding areas (170). The damage from stress (*i.e.,* extreme or chronic) results from over-activation of the hippocampal region by stress hormones (166). Human and animal studies have linked increases in glucocorticoids with reduced hippocampal volume, mostly from loss of synaptic connectivity or impairment of synaptic function, which also impaired performance on hippocampus-dependent memory tasks (e.g., 98; 151; 196; 198; 253). Glucocorticoid receptors found on the hippocampus reveal that this area is activated during the stress response and is involved in regulation of HPA axis activity (170). If there is atrophy or damage to this area, then the ability of the hippocampus to "shut off" the HPA axis in response to stress may be impaired (113; 122). Un-regulated or under-regulated HPA axis activity may result in prolonged HPA axis activation with excessive production of glucocorticoids and over-activation of the hippocampus, prefrontal cortex, and amygdala (212). In humans, reduced hippocampal volume has been linked to depression and PTSD (38; 128; 173). *To examine effects of physical and psychological stress on memory and the hippocampus, the present project included behavioral indices of memory and measures of activity in the hippocampus.*

In addition to stress hormone data, this work included data on associative memory. Associative memory can be assessed using the passive avoidance task (80; 192). This task is explained in the Behavioral Dependent Variables section (p. 48 ff.) of this proposal. *Data also were used to assess monoamine activity in the hippocampus. Using these variables, this project examined effects of stress on memory and the potential*

relationship between blast injury and psychological stress with a multivariate statistical approach.

Stress and Attention/Arousal

Stress leads to decreases in attention and increases in arousal. Specifically, stress has been associated with impaired attention (145) and activation of fear systems in the brain (174; 202). Similar to arousal, stress is associated with increased anxiety and PTSD (93; 99; 168; 202; 213). The prefrontal cortex (PFC) and amygdala, parts of the extended limbic and limbic system, are two areas of the brain important for attention and arousal (139) and associated with facilitating responses to stress (e.g., monoamine release in the brain; 14; 140; 194; 238). The limbic system has been implicated in anxiety disorders such as PTSD (114). Attention and arousal are psychological factors that are associated with PTSD, stress, and these brain regions. *This project included measures of attention/arousal and monoamine activity in the amygdala and PFC and examined relationships between these variables.*

Prefrontal cortex. The PFC is associated with executive function, the cognitive processes that regulate and control other cognitive processes including attention, working memory, and problem solving (109). The exact function of specific frontal areas is being identified as technology improves. The dorsolateral PFC has been associated with set shifting, problem solving, spatial information, working memory, and a supportive role in retention (9; 64; 210). The anterior cingulate cortex has been associated with emotional drives, experiences, integration, and inhibition of performance monitoring (142). The function of the PFC is disrupted during repeated or chronic stress, potentially due to inhibition or being overrun by subcortical areas (*e.g.,* amygdala). Ahima and colleagues

reported, like the hippocampus, the PFC contains receptors for adrenal steroids, though much less is known about effects of acute, repeated, or chronic stress on this region mediated via these chemicals (7; 8). Stress appears to cause atrophy in the PFC (e.g., shortening of dendrites; 42; 61; 170). The PFC has inhibitory inputs to the amygdala.

Amygdala. The amygdala is a part of the limbic system and the area of the brain associated with memory for emotional events and stimuli (109). Recently, the role of the amygdala in attention has been characterized using electroencephalography (EEG) recordings. Pessoa (189) reported the amygdala is involved in defining a stimulus and aiding in an appropriate response to a stimulus. Because of its classic role in emotional processing and recent understanding of its contributions to attention, the amygdala is clearly important with regard to the stress process. Experiments in animals indicate that stress also produces structural and functional changes in the amygdala. During stress and after repeated or chronic activation, this area may become over-activated. Excessive activation during stress appears to enhance growth in the amygdala (59; 245). Over-activation in the amygdala due to stress may be a result of excessive production of adrenal steroids because, like the PFC and hippocampus, the amygdala also has receptors for these biochemicals (7; 8). It also is possible that over-activation results because the amygdala is subject to regulation by frontal regions such as the PFC, which is inhibited by stress. Both routes to over-activation may be operating. Over-activation of this area in humans and animals has been linked with anxiety and PTSD, including enhanced fear responses (59).

Available evidence is consistent with the notion that stress inhibits the PFC and excites the amygdala (170), leading to problems with decreased attention and increased

arousal. Activation of the amygdala leads to release of monoamines throughout the brain (14; 140; 194; 238). *For this project*, *attention/arousal was measured using the acoustic startle reflex (ASR) and monoamine activity was collected from two nuclei of the amygdala (basolateral and central nucleus) and the PFC.* A detailed description of these methods is provided in the Dependent Variables section of this proposal (p. 48 ff.).

Dopamine. Attention and arousal also have been associated with the monoamine (more specifically, catecholamine) dopamine (DA). Dopamine's synthesis begins with the endogenous chemical tyrosine, converted to DOPA by tyrosine hydroxylase (TH), and further converted to DA by L-aromatic amino acid decarboxylase. The well-known neurotransmitter norepinephrine (a.k.a. noradrenaline) is synthesized from dopamine by an enzyme like TH, dopamine-β-hydroxylase. Even though DA is a precursor to norepinephrine, it was not identified as a separate neurotransmitter until almost a decade after norepinephrine was identified in peripheral nervous transmission (118). DA is metabolized in the brain, by monoamine oxidase (MAO) and aldehyde dehydrogenase, to form 3,4-dihydroxyphenylacetic acid (DOPAC) which is further metabolized by catechol-O-methyltransferase (COMT) to form homovanillic acid (HVA). See Figure 1 for an illustration of dopamine's metabolic pathway.

Dopaminergic neurons are organized into four functional neural networks (nigrostriatal, mesolimbic, mesocortical, and diencephalon). The mesocortical (midbrain areas to cortical areas) systems are made up of the inhibitory projections of the PFC to other cortical, limbic, and subcortical areas and are heavily involved in cognitive function, particularly attention and executive function (118). The present project involves data from the PFC, hippocampus, and amygdala, so the mesocortical system is

the most relevant to this proposal. Another DA system that is relevant to the present project is the diencephalon DA system, which is comprised of parts of the hypothalamus and thalamus that project to the spinal cord and plays a role in sensory and nociceptive processing (118). DA projections in this system connect DA and prolactin in that DA inhibits the release of prolactin from the pituitary (153). The functional significance of the relationship between DA and prolactin has yet to be fully understood. Given this system is involved in sensory processing and response, this relationship may be important in the study of stress. *DA activity in the brain was used in this project to determine its role in the stress response and how it interacts with the other chemicals collected.*

Stress increases monoamine transmission in the brain (69). Dopaminergic systems, which project into forebrain areas, all contain corticotrophin releasing hormone receptors (134; 150; 184). Stress, like some drugs of abuse, activates ventral tegmental area (VTA) dopamine neurons and stimulates limbic targets (105; 177). The idea that stress may act like a drug (*i.e.,* activates reward pathways) may seem contradictory, but it is possible that the activation of these areas by stress represents a positive, coping mechanism which represents a motivated response to handle the threat of the stressor (177). An alternative possibility is that stress is simultaneously activating other areas (*e.g.,* hippocampus or PFC) that contribute to the overall activation of the mesocortical dopamine system (249). The complexity of the stress response cannot be understood by conceptualizing neural networks or systems separately. Instead, they should be examined simultaneously and viewed in a different way from the traditional functional roles. *This doctoral project used multivariate data analysis techniques to examine the relationships*

among several peripheral and central physiological measures and behavioral indices of memory, attention/arousal, and pain.

Stress and Nociception

Nociception is the process by which the nervous system senses and then responds to noxious or damaging stimuli (147). The perception of painful stimuli begins with nociceptors or pain receptors, which become stimulated in response to chemical, thermal, or mechanical changes, and then pass an electrical signal through afferent pathways in the spinal cord to the brain, triggering autonomic responses to decrease the experience of pain, possibly including subjective or emotional responses(147). *This project included data from a measure of nociception, the hotplate procedure.* This procedure is described in detail in the Behavioral Dependent Variable section of this proposal (p. 48 ff.).

Serotonin (5HT). Stress increases serotonin transmission in the brain (69), most likely via CRF receptors located on serotonergic systems (134; 150; 184). Absence of glucocorticoid producing organs (adrenals) decreases serotonin neurons, and injection of glucocorticoids increases serotonin neurons in the raphe magnus (15-18). *This project examined 5HT activity in response to stress in brain regions relevant to memory, attention/arousal, and pain perception.*

Serotonin is a monoamine (more specifically, an indolamine) involved in integrating internal and external signals and responses, such as in pain and pain control (15; 16). The name serotonin was selected for this chemical because it was an agent acting in the serum to affect vascular tone (197). Serotonin synthesis is highly dependent on dietary intake of tryptophan, an amino acid that must be taken up into the central nervous system from the body. Only 1-2% of total body serotonin is in the brain; most is

found in the intestinal tract and immune cells and does not readily cross the blood brain barrier. Tryptophan is hydroxylized by tryptophan hydroxylase to form 5-hydroxytryptophan which is rapidly decarboxylated by aromatic amino acid decarboxylase to form 5-hydroxytryptamine (5HT) or serotonin (118). 5HT is further metabolized by monoamine oxidase and aldehyde dehydrogenase to form 5-hydroxy indole acetic acid (5HIAA). See Figure 2 for an illustration of serotonin's metabolic pathway.

Serotonin has a broad homeostatic function in the body. There is widespread serotonergic activity in the brain with many projections that coordinate complex sensory and motor functions (6). Given serotonergic function in the body is involved in integrating internal and external signals, is affected by stress, and affects many systems in the body, it is important to study how serotonin activity in brain areas relevant to TBI and PTSD is affected by psychological and physical stress. *This project measured serotonin activity in these relevant brain regions.*

5HT neurons occur as clusters of cells in/near the midline or raphe regions of the pons, upper brain stem, postrema, caudual locus coeruleus, in/around the interpeduncular nucleus. The caudal or posterior groups project to medulla and spinal cord, while the rostral neurons project to telencephalon and diencephalon. There also are extensive innervations by the intermediate groups (between the caudal/rostral) to the cerebral cortex. The group of neurons originating from the raphe medianus makes up a large component of 5HT innervation to the limbic system, while the dorsal raphe projects in greater density to neostriatum, cerebral cortex, cerebellar cortex, and thalamus. *This*

project used measures of 5HT activity at the ends of these projections (i.e., in the hippocampus, amygdala, prefrontal cortex, and insula).

Insula. The insula is an important brain area to consider with regard to perception of stress and response to painful stimuli and was included in this project. The insula is a brain region that has been associated with interoception. Interoception is the sensing of the physiological status of the body or representation of the body's internal state. The insula is involved in regulation of the internal state by maintaining homeostasis via initiation of motivated action (65-67). It is also activated during emotional memories (231) and is sensitive to a range of emotional stimuli, particularly those stimuli involving visceral representations (68; 71). Recent reports from brain imaging research studying PTSD suggest that the insula plays a role in traumatic memories, encoded via the dorsal visual stream, where the insula is activated representing emotional and bodily responses, versus the ventral visual system which would provide context about the event, not just the "feeling" of the event (39). Similarly, the insula is associated with increased activation during PTSD flashback memories (183), and reduced brain volume in the left insula is associated with increased flashbacks in PTSD (136). Flashback-associated increases in activation of the insula were evident in PTSD patients (compared to control and depressed group) showing significantly greater flashback-specific activity in the insula and other brain regions associated with emotion (250). *Monoamine activity in the insula was examined in the present research project.*

Sex Differences

There is a growing research literature that emphasizes sex differences in brain physiology. Neuroimaging techniques are revealing sex differences ranging from simple visio-spatial information processing (44), to differences based on the influence of sex

hormones (247), to differential activation after exposure to emotional stimuli (47). The concept of "functional connectivity" is being used to investigate brain sex differences as well (100). Functional connectivity represents the way in which brain areas that are not structurally connected communicate via neurophysiological events and is defined using correlations between these events (96). Functional connectivity has been assessed using *in vivo* functional neuroimaging techniques (e.g., EEG, MEG, BOLD fMRI, PET; 100). Many neurophysiological investigations have suggested that male and female brains communicate differently, especially in pathological states such as pain (144), migraine (146; 154), multiple sclerosis (216), and panic disorder (102; 180). Some investigators have suggested that sex differences in functional connectivity are only found under conditions in which the brain is active because, to date, male and female brains "at rest" appear to show similar functional connectivity (130). However, few studies have investigated the links between differences in brain function and sex differences in behavior and cognition (58; 100; 215; 248). *The present project investigated psychobiological responses in male and female brains to determine if males and females respond differently to stress and if these differences were related to sex differences in behavior.*

Study of bTBI and Stress

This doctoral dissertation project used data from a select set of variables, gathered from an experimental investigation of psychological and physiological effects of blast overpressure (BOP) induced traumatic brain injury (bTBI) and psychological stress in male and female rats, to examine effects of blast, stress, and blast + stress on behavioral (measures of attention, memory, nociception) and physiological (peripheral stress hormones and central neurotransmitters) responses. The author of this project was directly involved in the design of the experiment, collection of all behavioral and biological data, assays of biological samples, and data management. To date, the data collected in this experiment have yielded: presentation of a newly developed behavioral method (108), a master's project on sensory-motor and psychopathological responses to blast/stress (257; 259), and presentation of effects of blast/stress on central monoamine activity (22). In each of these cases, the dependent variables (DVs) were analyzed separately; no multivariate analyses on the DVs for this experiment have been conducted. Additionally, except for the central monoamine activity, each DV selected has not been the focus of any project or publication. Below is a detailed description of the experiment that provides the data used in the present project. Some of these details also appear in the Master's project of the author (257) and in a recent paper co-authored by the author of this project (260).

Experimental Design

The data used in this project come from an experiment conducted as a 2 (no blast, blast) x 2 (no stress, stress) x 2 (male, female) x 2 (1 day, 8 days post injury) full factorial mixed design. This experimental design resulted in 16 experimental conditions. In the

original project, there were eight or twelve subjects in each condition for a total of 143 rats. The number of subjects per condition was determined based on many years of animal experiments in the Grunberg Laboratory evaluating a variety of stressors and other independent and dependent variables similar to the variables examined in the experiment (4; 89; 106; 107; 254). Conditions were counterbalanced over seven cohorts for this experiment to ensure that historical and environmental factors would not confound experimental outcomes. *For the present project, 96 subjects were selected based on the data available for the central physiological measures. Based on preliminary analyses, it was determined by univariate ANOVAs that there were no significant differences between Time 1 and Time 2 on any behavioral or biological measures used in this doctoral project, so the dataset was collapsed across time. Additionally for this doctoral project, stress was conceptualized as having three experimental levels: 1) physical; 2) psychological; and 3) the combination. Additionally, based on preliminary univariate analyses and power analyses, it was determined that there were not enough subjects to test the interaction.*

Subjects and Housing

The subjects were 48 male and 48 female Sprague-Dawley (SD) rats from Charles River Laboratories (Wilmington, Massachusetts). Rats were approximately 54 days old upon arrival. This age was selected in consultation with the Neuroprotection and Modeling Group of the Center for Neuroscience and Regenerative Medicine (CNRM) at the Uniformed Services University and National Institutes of Health to be used in all TBI projects to model age demographics of Warriors. Investigators have determined that rat adolescence ends around 42 and 55 days for female and male rats, respectively (181;

227). Therefore, adulthood in rats is considered to begin around 60 days (86). The rats used in this experiment (54 days to 71 or 78 days) are comparable in age to young adult Warriors who have deployed in support of Operations Iraqi and Enduring Freedom (OIF/OEF). The SD strain was selected by the CNRM investigators because SD rats are extensively used in stress and brain injury research (55; 85; 88; 190; 199; 200; 223). Sex was one of the independent variables of this study, so male and female rats were included.

Rats were individually housed in standard polycarbonate shoebox cages (42.5 x 20.5 x 20 cm) with hardwood chip bedding (Pine-Dri). Subjects were individually housed to avoid any effects of social interaction and/or environmental enrichment on behavioral or biological variables (81; 82; 205; 206; 256). Cages were changed twice a week by Laboratory Animal Medicine (LAM) husbandry staff to ensure rats did not experience additional stress from excessively soiled housing conditions (191). Subjects had continuous access to standard, bland, laboratory chow (Harlan Teklad 4% Mouse/Rat Diet 7001) and water. The housing room was maintained at 23°C with 40% relative humidity on a 12 hr reverse light cycle (0600-1800 lights off). To measure rats during their active time, a reverse light cycle was used. Rats are nocturnal animals, with normal high-activity during dark periods. The experiment was conducted under a protocol approved by USUHS Institutional Animal Care and Use Committee (MPS-09-732; Biobehavioral assessments of traumatic brain injury in rats) and conducted in full compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (50).

Independent Variables (IVs)

Stress manipulation. Uniformed service members are exposed to stressful, life-threatening and unpredictable situations in the combat environment (125). The Warrior Stress Paradigm (WSP) for rats was created to model the anticipatory stress commonly experienced by Warriors as they prepare for and engage in life-threatening missions in hostile, deployed settings (258). This model is based on previous studies conducted in the Grunberg laboratory (29; 110; 149; 188; 229; 257) and based on other reports (e.g., 111; 252). This method uses a combination of a predator stressor and unpredictable environmental stimuli.

Predator stress is a non-painful, but effective stressor used in rodent models investigating the effects of stress, which is manipulated by presenting the actual predator or the odors of the predator. Exposure to predator stress produces behavioral changes in rodents including changes in food consumption, anxiety-like behavior, startle response, freezing behavior, withdrawal behavior, and exploratory behavior (28; 29; 83; 110; 133; 149; 158; 188; 203; 229; 237; 257) and increases in stress hormones (29; 46; 111). In this experiment, predator stress was presented by placing a cotton ball with commercially available, synthetic fox urine (Buck Stop, Stanton, MI) into a test cage with the rat subject.

Unpredictable, non-painful stressors are included in the WSP to model environmental stress experienced by Warriors. These procedures also are included to avoid habituation to repeated presentation of the fox urine. Unpredictable stressors (*e.g.,* noise, flashing light, and cage shaking) in rodent studies provide a face-valid model of

human stress that reliably produce alterations in behavior (29; 95; 101; 110; 149; 188; 203; 229; 257) and increases in stress hormones (95; 246).

The stress procedure lasted for 20 minutes each day it was used. Animals in the stress condition were transferred, individually, from their home cages to separate "stress cages" (29 x 18 x 12 cm) with a lid and no bedding (see Figure 3). A large cotton ball, drenched with fox urine (15mL) was placed in varying spots in the stress cage. The procedure was conducted in a room separate from the housing room and the behavioral measurement rooms. A bright, florescent, overhead light remained on during the procedure. The stressors were administered for seven consecutive days in a manner designed to minimize habituation of the stress response. Figure 4 presents the WSP timeline. Animals in the non-stressed conditions remained in the housing rooms during stress manipulations.

Animal Models of TBI and Blast overpressure (BOP). There are several paradigms available to study TBI in animals (*i.e.,* fluid percussion, controlled cortical impact, and weight drop). The fluid percussion model, described in detail by Ling and colleagues (143), replicates contusion without skull fracture. The controlled cortical impact (CCI) paradigm is used to produce a focal brain injury, and was developed to allow for better control over biomechanical factors (*e.g.,* velocity of impact and depth of deformation) than offered by the fluid percussion model (57; 78). The CCI usually involves craniotomy (*i.e.,* opening the skull) and does not model a diffuse brain injury. The weight drop paradigm is used to model constrained impact-acceleration head injury and reliably produces graded diffuse axonal injury (52; 92; 156). This method requires physical contact with the head and, therefore, it is limited for modeling blast-induced

brain injury. These methods do not model brain damage from blast exposure; *i.e.,* they do not model brain damage from exposure to IEDs.

The BOP method of blast exposure is designed to resemble the conditions to which Troops are exposed from IED blast on the battlefield (148). The BOP method can be used to produce different air-blast levels that are survivable to inflict primary blast injury, and to study effects of blast on the brain (55). To model human mild TBI, which is associated with diffuse axonal injury (13; 175), the air-blast exposure of ~100 kPa was used in the present experiment. Blast exposure of ~100 kPa produces a diffuse injury, while lower levels produce no significant damage, and higher levels result in greater total cell damage and loss (148).

Blast-induced brain injury can be inflicted using the BOP paradigm, which models primary blast exposure. Functional, biochemical, and morphological changes in the brain are initiated by the primary pressure from a blast wave (53), but it is not clear how the primary blast wave injures the brain (55). Experiments using blast models have revealed the following biological and behavioral effects: blood brain barrier breakdown (200), increased intracranial pressure (211), brain edema (24), increased time on balance beam (148), impaired sensory-motor reflexes and neurobehavioral functioning (see Figure 5; 200; 257), and impaired spatial learning and memory (135; 148).

Rats in the blast TBI experimental groups were transported to Walter Reed Army Institute of Research/Naval Medical Research Center (WRAIR/NMRC) Forest Glen and back to USUHS in a closed van provided by USUHS. Rats were housed in cardboard transportation cages which were secured from sliding during transportation, and room temperature was maintained while being transferred from the van to the BOP laboratory.

Rats were transferred to a holding area separated from the BOP laboratory by a cinderblock wall and a sound attenuating wall. The BOP paradigm was conducted in accordance with the methods described by Yarnell et al. (260). Immediately prior to the blast exposure, rats were individually placed into a polystyrene container and anesthetized (5% isofluorane mixed with oxygen at 1L/min for 2 min) before exposure to BOP (54). Each anesthetized rat was placed into a metal, mesh basket and secured with two rubber straps (see Figure 6). Rats' bodies were oriented facing the origin of the blast. This orientation provides frontal exposure which results in higher amplitudes and longer durations of the pressure waves in the brain compared to side-on orientation (55). The basket was then placed at the mouth of the blast tube. The blast tube is a horizontally mounted, 12-inch diameter, circular, 19.5 ft long, steel tube (see Figures 7 and 8). The tube is divided into a 2.5 ft compression chamber separated from a 17 ft expansion chamber by polyethylene MylarTM sheets (0.0254 cm thick; Du Pont Co., Wilmington, DE). Pressure increases in the compression chamber until the Mylar sheet ruptures and generates a pressure wave that produces an exposure pressure of ~100 kPa for less than a second.

After injury, the rats were returned to their transport cages, monitored until consciousness was regained (approximately 5 min), transported back to the USUHS LAM housing facility, and returned to their home cages. Rats in the non-injured experimental conditions remained in the animal housing rooms at USUHS.

Sex. Few experimental investigations have compared psychobiological effects of different levels of stress in male and female rats. Sex differences were investigated in

this project because males and females may have different biological, neurophysiological, and psychological/behavioral responses to stress (49; 100; 240).

Dependent Variables (DVs)

There were several DVs in this experiment: eight behavioral measures; levels of three stress hormones; levels of three neurotransmitters and their metabolites from eight different brain regions; seven immune system markers; and structural brain imaging on a select set of subjects. Figure 9 presents a timeline of the parent project and Table 1 provides a list of all measures conducted.

The DVs analyzed in the present project were selected based on theoretical conceptualization and literature review of variables relevant to psychological and physiological responses to stress. The DVs used in this doctoral project included: behavioral measures to assess memory, attention/arousal, and pain; levels of peripheral stress hormones (corticosterone, ACTH, prolactin); and monoamine (dopamine, serotonin) activity in five brain regions relevant to the behavioral measures (prefrontal cortex, basolateral amygdala, central nucleus of the amygdala, hippocampus, insula cortex).

Behavior. The three behavioral measures used in the present project included measures of memory, attention/arousal, and pain. These psychological concepts were selected because they represent three of the key post-deployment symptoms experienced by Warriors with TBI and/or PTSD (251). The author was involved in the collection of all behavioral data, the training and supervision of research assistants and graduate students working on the project, and management of collected data.

Memory assessed using passive avoidance. The passive avoidance paradigm provides an index of memory (74; 79; 80; 201). This paradigm is based on assessing the animal's ability to inhibit its natural tendency to move from a lit to dark space, when the dark space is associated with a painful stimulus (mild shock). An animal that delays crossing from the lit to dark space "remembers" the shock; in contrast, an animal that crosses without delay, does not "remember" the shock.

Passive avoidance was trained and tested in two Med Associates shuttle boxes (EVN-018MD, St. Albans, VT). The apparatus consists of two boxes (21 cm x 25 cm x 17 cm each) separated by a mechanical door (see Figure 10). The rats were first trained to avoid the dark compartment. On training day, the rat was placed in the lit box (50 watt bulb). After 1 minute acclimation, the door separating the boxes opened. Once the rat crossed into the dark box, the door closed and a 0.8 mA shock was administered through the metal grid floor for 5 sec. The rat was tested 24 hours later, and its latency to cross into the dark box was measured. An increase in the amount of time (in sec) to cross into the dark chamber reflects memory of the shock. If the rat did not cross into the dark box, then the test was stopped after 5 min. Rats were trained either 2 or 9 days post-injury. Rats were tested either 3 or 10 days post-injury. *The test latencies were used in the data analysis of the present project.*

Attention/arousal assessed using acoustic startle reflex (ASR). The acoustic startle reflex (ASR) is a set of unconditioned behaviors (*e.g.,* involuntary, defensive, muscular responses) to sudden, intense acoustic stimuli that is believed to index central information processing and possibly attention (2; 3; 5; 72; 88; 90; 233). For example, a person may "jump" after hearing a loud noise (*e.g.,* air horn) in an otherwise quiet room,

but then "recovers" quickly. This behavioral example of "surprise" is an appropriate reaction to the unexpected stimuli. This reflex, present in humans and rats, is considered to be an index of reactivity to external auditory stimuli. Because the reflex can be elicited using the same stimuli across species, the ASR paradigm can be generalized from the animal model to human responses (232). Exaggerated startle responses (behavioral, physiological) can be elicited following exposure to stress in rodent studies (220) and have been associated with PTSD in humans (224).

The brainstem and other non-brainstem structures, including the hippocampus, periacqueductal gray, median raphe, and inferior colliculus, are involved in the startle responses, (33; 45; 62). Given the involvement of these structures, the reflex also can be altered by higher level processes such as attention (11; 226) and emotion (37; 137).

Acoustic startle reflex amplitudes were measured in a Med Associates Acoustic Response Test System (Med Associates, Georgia, VT). The Acoustic Response Test System consists of weight-sensitive platforms inside individual sound-attenuated chambers (see Figure 11). Subjects' movements in response to stimuli are measured as a voltage change by a strain gauge inside each platform. Responses are recorded by an interfaced Pentium computer as the maximum response occurring during the no-stimulus periods and during the startle period.

Each rat was individually placed in a ventilated holding cage. The holding cages are small enough to restrict extensive locomotion but large enough to allow the subject to turn around and make other small movements. Each cage was then placed on a weight-sensitive platform. A ventilating fan built into the chamber provided background noise.

Following placement of animals in the chambers, a 3-minute adaptation period was conducted during which time no startle stimuli were presented.

Startle stimuli consisted of 110 or 120 dB noise bursts of 20 msec duration. Decibel levels were verified by a Larson-Davis Sound Pressure Machine Model 2800 (unweighted scale; re: 0.0002 dynes/cm^2). Each startle stimulus had a 0 msec rise and decay time so onset and offset were abrupt, a primary criterion for startle. There were multiple types of stimulus trials, and each trial type was presented eight times. Trial types were presented in random order to avoid order effects and habituation. Inter-trial intervals ranged randomly from 30-60 sec. Trial types were: (1) 120 dB stimulus, or (2) 110 dB stimulus. The testing period lasted approximately 25 min. Holding cages were washed with warm water and dried after each use. Males and females were tested in separate test chambers.

Recordings of subject movements during no-stimulus periods were used to control for movements on the platform not related to the startle stimulus. For data analysis purposes, each animal's responses were averaged within trial type. These calculations are based on established procedures of several investigators (1; 2; 45; 232-234). Two acclimation periods were conducted prior to taking baseline measurements (87). ASR measurements were recorded two times: before any experimental manipulation (baseline), and either 2 or 9 days after experimental manipulations began (*i.e.,* after injury and/or during stress). *The measurement taken after experimental manipulations was used in the data analysis of the present project.*

Nociception assessed using hot plate. Nociception is the process of sensing and responding to noxious stimuli (147). The hot plate test measures the latency of a rat to

lick its hind paw in response to a noxious, thermal stimulus. The hind paw lick is a supraspinally mediated response (*i.e.,* not a peripheral reflex) to the aversive stimulus and involves sensory and motor cortical areas in the brain (77; 193).

The test was conducted using the Omnitech Hot Plate Analgesiometer (HP, Omnitech Electronics, Inc. Columbus, Ohio) using a well-established procedure (12; 193). The animals were placed in the center of the apparatus, consisting of a 25 x 25 cm metal plate heated to 51° C, consistent with previous procedures (12; 193) and enclosed by Plexiglas on all sides (see Figure 12). The latency of the nociception response to the heated plate was recorded to the nearest one thousandth of a second. The rat was removed after it licked its hind paw or after 60 sec. The hot plate was cleaned between trials to remove debris or waste. The animals were tested prior to injury (baseline). Post-injury testing occurred at 3 or 10 days post injury depending on treatment group. *The pain sensitivity at 3 or 10 days was used in the data analysis of the present project*.

Central physiology. The central physiological measures used in the present project included dopamine and serotonin activity in the prefrontal cortex (PFC), basolateral amygdala (BLA), central nucleus amygdala (CNA), hippocampus (HIP), and insula cortex (IC). These neurotransmitters and brain regions are relevant to memory, attention/arousal, and pain (see Psychobiological Effects of Stress section) -- three psychological concepts key to post-deployment symptoms experienced by Warriors with TBI and/or PTSD and which also are included as DVs.

Euthanasia. Rats were euthanized by decapitation following carbon dioxide-induced anesthesia, in accordance with USUHS LAM-approved current procedures. The author planned, supervised, and participated in the euthanasia of each cohort. The brain

was removed from the rat, placed in a Jacobowitz brain block (see Figure 13), and cut caudal to the interpeduncular nucleus at the level of the cerebellum for easier access to the brain regions of interest. Samples were immediately frozen using dry ice to preserve samples and to prevent breakdown of biological material, then placed into scintillation valves, and frozen until slicing. Samples were later sliced, micro-dissected, assayed for total protein, and then assayed for monoamines.

Brain slicing. Brain slicing and micro-dissection are sophisticated techniques developed by Palkovitz (185) and adapted by Jacobowitz (120). Dr. David Jacobowitz was a collaborator for this experiment. The author assisted Dr. Jacobowitz with these techniques by preparing, organizing, and managing samples.

Brains were sliced using a microtome inside a -6°C Cryostat. For optimal protein punching per region, approximately thirty slices were made at 300 µm and adhered to a 5 x 7.5 cm glass slide by briefly thawing the slices on the slides (see Figure 14). These slices were then stored at -80°C in an aluminum wrapped slide box until micro-dissection (120; 121; 185; 186).

Micro-punching. The following five brain regions were selected from the total of eight collected in the original experiment, because they regulate emotion, reasoning, pain, memory. Brain Regions (Prefrontal Cortex [PFC], Insula Cortex [IC], Hippocampus [HIP], Basolateral Amygdala [BLA], Central Nucleus Amygdala [CAN]), were punched using stainless steel cannulae (500, 700, or 1000 µm in diameter) and a -10°C to -5°C dissection cold plate (FTS System, RT209 NY; 120; 121; 185; 186). Coordinates of specific brain regions were identified using a rat brain atlas. A light source microscope (resolution of 1.2 x) was used to enhance view of the brain slices (Ehrenreich Photo

Optical, MK II Fiber Optic Light). After micro-dissection, the samples were placed into 1.5 mL Eppendorf tubes containing 50 µL of 0.1 N perchloric acid with 100 µM EDTA to preserve the tissue from degradation. The tissues were combined with the solution briefly (~2 sec), spinning the tube using a microfuge. Samples were then stored at -80°C to prevent breakdown of biological materials until protein assays were conducted.

Protein assay. Because brain regions differ in size and amount of tissue and because of the nature of the HPLC analysis (explained below), the amount of total protein concentration was first determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, CA) using bovine serum albumin standard (36). These assays also were conducted in Dr. Jacobowitz's laboratory, and the author assisted by organizing samples, calculating the standard curve for the assay, recording concentration readouts, and managing the collected data.

Samples were thawed and organized according to brain region. Some of the brain regions are large (*e.g.,* PFC), whereas others are very small (*e.g.,* BLA and CNA). Because there are differences in the amount of tissue depending on brain region, the amount of material used for the assay differed for each brain region from 1- 10 µL (1µL- HIP; 2 µL- PFC, Insula; 10 µL- BLA, CAN). The samples with greater amounts of tissue (PFC and HIP) required an additional 50 µL of 0.1 N perchloric acid with 100 µM EDTA preservative be added before the assay. Samples were thawed to room temperature (23°C) and then sonicated for three, 1 second intervals, placed in 2 ml plastic cuvettes containing Bio-Rad reagents, and mixed. Individual cuvettes were placed in a UV/Vis spectrophotometer to determine the absorbance of the solution in the cell by passing light through the mixture (turbidity). Amount of protein in the sample was calculated using

the standard curve for known solutions and was expressed as a concentration in µg/µl. This value was then used in the calculation for each transmitter described below to account for differences in amount (weight) of the tissues assayed.

HPLC. Concentrations of monoamines and their metabolites were quantified using high-performance liquid chromatography with electrochemical detection (HPLC-ECD) for each microdissected brain region. These neurochemical analyses were conducted in the laboratory of Dr. Michael Baumann at the National Institute on Drug Abuse (NIDA). The author organized and prepared all samples for transport to NIDA and managed all data collected from HPLC analyses.

Tissue punches from individual rats were homogenized in 100 µL of ice cold 0.1 N perchloric acid with 100 µM Na_2EDTA and spun at 15,000 rpm for 15 min in a refrigerated microfuge (Eppendorf Model 5415R, Eppendorf North America, Hauppauge, NY) set at 4^{o}C. Concentrations of dopamine (DA) and its metabolites (3,4-dihydroxyphenylacetic acid [DOPAC] and homovanillic acid [HVA]), and serotonin (5-HT) and its metabolite (5-hydroxyindoleacetic acid [5-HIAA]) were quantified in the supernatant using HPLC-ECD (21; 23; 225; 229). Briefly, 20 µl aliquots of the supernatant were injected onto a C18 ODS 5 µm column (4.6 x 250 mm) linked to a coulometric detector (Model Coulochem III, ESA-Dionex, Chelmsford, MA, USA). The aliquots of supernatant were delivered to the column via a computer-controlled autoinjector module (Model 2707, Waters Corp., Milford, MA, USA). Mobile phase consisting of 50 mM sodium phosphate monobasic, 250 µM Na_2EDTA, 0.03% sodium octanesulfonic acid, and 25% methanol (final pH=2.75) was recirculated at 0.9 ml/min using a dual piston HPLC pump (Model 515, Waters Corp.). Data were acquired using

Empower II software (Waters Corp.), and peak areas of unknowns were compared to those of standards. A standard curve containing all analytes was run prior to each set of 32 samples to ensure analyte retention times and standards were linear from 30-1000 pg. The lower limit of assay sensitivity (*i.e.,* 3x baseline noise) was 10 pg per sample for each analyte (21; 23; 229). The amount of sample in each brain region for each metabolite was given in pg. To determine the concentration of the metabolite in a brain region (in pg/μg protein), the sample amount was divided by 20 μl (amount injected onto the column) and then divided by the protein concentration (described above) in the given brain region. To estimate "monoamine activity" or the amount of each neurotransmitter "being used" or "converted" in each brain region, a ratio was created of metabolite to transmitter for dopamine ([DOPAC + HVA]/ DA) and serotonin (5HIAA/5HT). *The ratio values were used in the data analysis of this doctoral project.*

Peripheral stress hormones. The author supervised and participated in the collection and management of all blood samples, the organization and preparation of samples for assay, and data management following biological assays. The assays were conducted by a technician in the Grunberg laboratory. Trunk blood was collected immediately following decapitation of the rats into a 7 ml BD Vacutainer tube with Sodium Heparin (Becton Dickinson, Franklin Lakes, NJ), and was placed directly into wet ice to prevent clotting. Blood samples were centrifuged at 4°C and 2,400 rpm for a total of 15 minutes (International Equipment Company, Needham Heights, MA). Once centrifuged, the plasma (supernatant) of each blood sample was aliquoted into Eppendorf tubes (100 μl each). Samples were stored in a -80°C freezer until assayed (Thermo Electron Corporation Forma -86°C ULT Freezer, Waltham, MA).

Corticosterone assay. Corticosterone is a glucocorticoid steroid hormone in rats, like cortisol in humans, that is produced and secreted from the adrenal cortex in response to the presence of adrenocorticotropic hormone (ACTH) in the blood. Corticosterone was used as a biomarker of stress for this experiment based on the fact that glucocorticoid production is increased by stress (40; 41; 103; 222).

Corticosterone levels were measured using Cayman's Corticosterone Enzyme Immunoassay (EIA) Kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instructions with minor adjustments made, such that the plasma samples thawed for an hour in a 50°C water bath using the Fisher Scientific ISO Temp 202 water bath (Fisher Scientific, Dubuque, IA) to incubate, in order to mitigate protein interference with the hormones within the samples. A dilution plate was run prior to the experiment to determine the most appropriate dilution for optimal results with the assay. Within the dilution plate, three different dilutions (1:500, 1:1,000, 1:10,000) were considered to set-up and test in duplicates with plasma samples from each represented cell group. Plasma samples were diluted 1:100 and measured in duplicate. Each EIA plate was organized with pre-designated wells for the standards and non-sample reagents. All other remaining wells were utilized for sample assay use, and were strategically organized within the wellplate, such that each cell group was represented at least once. *Levels of CORT (ng/ml) were used in the data analysis of this doctoral project.*

Prolactin assay. Prolactin is a peptide hormone (luteotropic hormone, LTH) produced and secreted by the pituitary gland. Prolactin is known to physiologically increase the stimulatory effect of ACTH-induced corticosterone secretion in rats (123).

Prolactin levels were measured using SPI-Bio/Bertin Pharma's Prolactin Enzyme Immunoassay (EIA) Kit (SPI-Bio/Bertin Pharma, Montigny le Bretonneux, France) according to the manufacturer's instructions with minor adjustments made, such that the plasma samples thawed for an hour in a 50°C water bath using the Fisher Scientific ISO Temp 202 water bath (Fisher Scientific, Dubuque, IA) to incubate, in order to mitigate protein interference with the hormones within the samples. A dilution plate was run prior to the experiment to determine the most appropriate dilution for optimal results with the assay. Within the dilution plate, two different dilutions (1:2, 1:4) were considered to set-up and test in duplicates with plasma samples from each represented cell group. Plasma blood samples were diluted 1:4 and measured in duplicate. Each EIA plate was organized with pre-designated wells for the standards and non-sample reagents. All other remaining wells were utilized for sample assay use, and were strategically organized within the wellplate, such that each cell group was represented at least once. *Levels of prolactin (ng/ml) were used in the data analysis of this doctoral project.*

Adrenocorticotropic hormone (ACTH) assay. Adrenocorticotropic hormone (ACTH) is an amino-acid peptide hormone produced and secreted by the pituitary gland. ACTH regulates the production of steroid hormones (from the adrenal cortex) by increasing synthesis and secretion of adrenal steroids, aldosterone, cortisol, and adrenal androgens. ACTH production and secretion functions in feedback with cortisol (glucocorticoid) levels, such that increased cortisol levels inhibit ACTH production and secretion, and decreased cortisol levels elevate ACTH production and secretion.

ACTH levels were measured using Calbiotech's ACTH Enzyme Immunoassay (EIA) Kit (Caltbiotech, Inc., Spring Valley, CA) according to the manufacturer's

instructions with minor adjustments made, such that the plasma samples thawed for a hour in a 50°C water bath using the Fisher Scientific ISO Temp 202 water bath (Fisher Scientific, Dubuque, IA) to incubate, in order to mitigate protein interference with the hormones within the samples. A dilution plate was run prior to the experiment to determine the most appropriate dilution for optimal results with the assay. Within the dilution plate, four different dilutions (1:10, 1:20, 1:50, 1:100) were considered to set-up and test in duplicates with plasma samples from each represented cell group. Plasma blood samples were diluted 1:10 and measured in duplicate. Each EIA plate was organized with pre-designated wells for the standards and non-sample reagents. All other remaining wells were utilized for sample assay use, and were strategically organized within the wellplate, such that each cell group was represented at least once. *Levels of ACTH (ng/ml) were used in the data analysis of this doctoral project.*

Specific Aims and Hypotheses

Specific Aim 1

The first specific aim was to examine psychobiological variables gathered from an experimental investigation of Warrior stress to identify strongly related variables. It was important to determine whether psychological constructs (*i.e.,* memory, attention/arousal, pain) can be characterized by sets of strongly related psychobiological variables that are affected by stress. Consideration of variables using a multivariate approach may enhance understanding of the stress process and may help elucidate mechanisms that may lead to improved understanding and treatment of stress-induced injuries, including bTBI and PTSD. The three hypotheses described under Specific Aim #1 were based on conceptual and empirical findings reviewed in the background section of this doctoral dissertation.

Hypothesis 1a. Psychobiological variables associated with memory (*e.g.,* passive avoidance score, dopamine activity in hippocampus, corticosterone) are strongly interrelated. It was hypothesized that variables associated with memory would be related to each other because: passive avoidance is a way to measure memory in rodents (80); the hippocampus is the primary brain region associated with memory formation (109); corticosterone is increased by stress (109); the hippocampus contains receptors for stress hormones that can be affected by stress (170).

Hypothesis 1b. Psychobiological variables associated with attention/arousal (*e.g.,* acoustic startle score, monoamine activity in the amygdala and prefrontal cortex) are strongly interrelated. The rationale for Hypothesis 1b was that the behavioral

variables used to assess the psychological construct of attention/arousal would be related to underlying physiological activity in brain areas related to this psychological construct.

Hypothesis 1c. Psychobiological variables associated with pain (*e.g.,* hotplate score, serotonin activity) are strongly interrelated. The rationale for this hypothesis is similar to 1a and 1b in that, it was expected that the behavioral variables would be related to underlying physiological measures.

Specific Aim 2

The second specific aim was to determine if there was a difference in the relationships among the psychobiological variables identified in Specific Aim #1 between levels of stress (*i.e.,* psychological stress, physical stress, and the combination of psychological and physical stress) and sex (male, female), and if combinations of the psychobiological factors discriminate membership in one of the stress levels or sexes. Because the conditions of TBI and PTSD have overlapping symptoms, it was important to determine whether sets of strongly related psychobiological variables identified in Specific Aim #1 are differentially affected by types of stress (*i.e.,* psychological, physical, or combination). This determination may help improve diagnosis and treatment of stress-induced injuries. In addition, because males and females may respond differently to stress, it was important to consider sex differences in these analyses. The four hypotheses described under Specific Aim #2 are based on concepts and findings described in the background section of this doctoral dissertation.

Hypothesis 2a. Psychobiological variables that are strongly associated with memory (see Figure 15) best predict group membership in the psychological stress group only. The rationale for this hypothesis was that psychological stress would have the

greatest impact on memory variables because adrenal responses are primarily anticipatory in nature and brought on by psychological stressors (161) and excessive activation by glucocorticoids would disrupt function in the hippocampus (170).

Hypothesis 2b. Psychobiological variables associated with attention/arousal (see Figure 16) best predict membership in the combination of physical and psychological stress condition. This hypothesis was based on the human TBI and PTSD literature that revealed associations between TBI and functional difficulties including missed work days, sleep problems, and problems with concentration, were somewhat mediated or moderated by the occurrence of PTSD and depression (26; 117; 251).

Hypothesis 2c. The psychobiological variables that are associated with pain (see Figure 17) best predict membership in the physical stress (*i.e.,* blast exposure) group. This hypothesis was based on the human TBI literature that revealed the only symptoms that remained associated with TBI after controlling got PTSD and depression were somatic in nature and related to pain including headache (117; 251) and arm, leg, and joint pain and chronic pain (251).

Hypothesis 2d. Different combinations of psychobiological variables discriminate between male and female rats and stress affects male and female rats differently. It was hypothesized that stress would affect males and females differently based on a growing literature of differential stress responses in males and females (e.g., 240) and other behavioral findings from this experiment where males and females showed different patterns of responses to stress, such that stress and the combination of blast + stress had greater deleterious effects on female behavioral responses than on male responses (257).

Statistical Approach and Methods of Analyses

This research project used data gathered from an experimental investigation of psychological and physiological effects of blast overpressure (BOP) induced traumatic brain injury (bTBI) and psychological stress in male and female rats described in the previous sections. This project used a comprehensive and sophisticated data analytic strategy to examine complex relationships among the behavioral and biological data collected. The data analytic strategy was designed as a series of analyses: (1) to identify strongly related psychobiological variables; (2) to use this reduced number of variables to determine if combinations of psychobiological variables are differentially affected by stress (psychological, physical, or a combination of psychological and physical stress); and (3) to determine if male and female rats differ on combinations of psychobiological variables. The possibility of Type I error was reduced by using Exploratory Factor Analysis to decrease the number of variables to be used in subsequent analyses and then by conducting multivariate analysis of variance (as opposed to multiple univariate analyses). Possibility of Type II error was managed by conducting power analyses. The following sections present the statistical analyses that were conducted.

Step-wise Statistical Approach

Statistical analyses were conducted in a step-wise manner. The first step was to reduce the set of 16 psychobiological variables (see Table 2) to a smaller number of strongly related variables. This step was accomplished using exploratory factor analysis (EFA; details are presented below). The second step was to determine if different levels of stress have differential effects on the reduced number of related variables (ideally factors created in step #1), and if psychobiological variables are different for male and

female rats. This step was accomplished by entering the factor scores, estimated using EFA, into a multivariate analysis of variance (details are presented below).

Two alternative approaches to analyzing the data were conducted to determine the best approach for analyzing multivariate data. The first approach was to conduct a multiple discriminant analysis (MDA) to determine if stress groups can be differentiated based on combinations of psychobiological variables. Details of the discriminant analysis are presented below. The second approach to determine how stress affects combinations of psychobiological variables was to examine separate, multivariate models with MANOVAs built based on conceptualized relationships in the data set. Details of this approach are presented below.

Factor Analysis

The first specific aim was to examine psychobiological variables, gathered from an experimental investigation of Warrior stress, to identify strongly related variables. The first step was to examine a set of psychobiological variables and extract the most strongly related linear combinations of these variables using exploratory factor analysis (EFA), with SPSS Version 20 software. Because these psychobiological variables are inherently related (*i.e.,* come from the same animal) and there is support in the literature that they are mutually regulatory (159; 161; 162; 164; 167; 170), it was hypothesized that underlying constructs exist in the data that are not evident using separate analyses of each dependent variable related to distinct psychological concepts (*e.g.,* memory, attention/arousal, pain). These underlying patterns in the data can be made apparent with EFA which extracts these latent variables from the data in a systematic and rigorous way.

Factor analysis, originally developed by Spearman (228), is a statistical technique conducted to simplify complex sets of data to determine the constructs or dimensions that account for the correlations between variables (132). Correlations are a numerical measure of the degree of relationship between two sets of scores (with a range between -1 to +1). A correlation of 0 indicates no relationship exists, whereas correlations of ±1 indicate a perfect, positive or negative relationship. When a number of variables are considered together, a correlation matrix (or a set of correlation coefficients) is produced. In the present work, 16 variables were considered (see Table 2) which resulted in 256 entries into the correlation matrix. When clusters of high correlation coefficients are found in a data matrix, it is possible that those correlated variables are measuring some underlying dimension. Factor analysis can be used to simplify this matrix by extracting a smaller number of factors that can account for the correlations in the dataset.

Deriving factors. A factor can be defined as a dimension or construct which represents a relationship among a set of variables (132; 207). In the EFA method, factors are derived directly from the data included in the model and all variance is accounted for, including variance due to error (a.k.a. unwanted or unsystematic variance). Because of the iterative nature of EFA, it maximizes the amount of variance explained by any number of factors. However, it is possible that EFA is contaminated by error, and the factors derived should only be used to describe the latent structure of the data matrix, and should not be used to make generalizations outside the data set. EFA was used in this project to describe the latent structure of the data matrix and to inform the development of multivariate models.

Factor selection. There is debate over how to select the number of factors to be retained. Generally, criteria are based on factor loadings and eigenvalues. A factor loading represents the correlation between the observed score on a variable with the factor generated by EFA. A factor loading is calculated using eigenvalues. Eigenvalues and eigenvectors are used to describe the dimensions or shape of the relationship between variables that shows the distribution of variance in a matrix (91). Factor loadings of > 0.6 are considered "high" and factor loadings between 0.3 and .6 are considered "moderately high." Factor loadings of < 0.3 indicate no relevant relationship between the variable score and the created factor. The classic method to determine factor retention is based on the size of the eigenvalue and use of a scree plot, *i.e.,* a graph with each eigenvalue (Y-axis) plotted against its associated factor (X-axis; 51). However, this method is only considered reliable when there are more than 200 subjects included in the dataset (91), so it was not appropriate for this project which had an N of 96.

Another method is to use all factors with eigenvalues >1 (126). This method may overestimate the number of factors to retain, but is considered accurate when there are < 30 variables in a dataset and communalities are > 0.7 (91). The present project had 16 variables in the dataset and used EFA, which assumes all variance is shared and so communalities are equal to 1. Communality is the proportion of common variance present in a variable. If a variable has no unique variance, then its communality is 1; if a variable has no common variance or a lot of specific error variance, then its communality is 0. EFA assumes all variance is shared variance and, therefore, the communality for each variable is 1 and all factors are retained. To identify if common variance really exists in the dataset, some factors may need to be discarded. When this is the case, the

resulting communalities are <1 and provide information about how much variance is explained by the remaining factors. Once the factors have been extracted, a new communality value is calculated (by squaring and summing the rows of a factor matrix) to determine the amount of variance that is explained by the extracted factors.

Rotation. EFA was used in this project to identify strongly related variables in the dataset. It was hypothesized that groups of variables related to memory, attention/arousal, and pain would become evident as separate factors using EFA. Once factors were derived, then the degree to which variables "load," or are correlated with a factor, was calculated. Because of the iterative nature and algebra of deriving factors, most variables have erroneously high loadings for the first (or strongest/most important) factor and lower loadings on all others (91). To accurately interpret the factor scores and use these numbers in subsequent analyses, a rotation of the factors was conducted. Factors can be rotated using two methods: orthogonal and oblique. Orthogonal rotation keeps factors independent/uncorrelated and oblique rotation allows the factors to correlate during rotation. Oblique rotation was used in this project because factor loadings were used in subsequent multivariate models which assume some amount of correlation (*i.e.,* not too high, not too low) between variables.

Multivariate analysis of variance (MANOVA)

The second specific aim was to determine if there is a difference in the relationships among the psychobiological variables identified in specific aim 1 for different levels of stress (*i.e.,* psychological stress, physical stress, and the combination of psychological and physical stress) and sex (male, female), and if combinations of the psychobiological factors discriminate membership in one of the stress levels or sexes.

This aim was addressed using a 4 x 2 multivariate analysis of variance (MANOVA). The first independent variable (IV) was stress with four levels: control, psychological stress, injury, and psychological stress + injury. The second IV was sex with two levels: male and female. The factors (with factor scores), identified in the EFA, were used as the DVs. An alpha level of .05 with two tails was used.

MANOVAs are a type of general linear modeling technique that can be used to detect group differences for several dependent variables (91). This technique can be used to: account for the relationship between dependent variables; determine whether groups differ along a combination of dimensions; and determine whether groups of subjects can be identified by a combination of scores on outcome variables (91). MANOVAs are constructed based on theoretical and/or empirical bases, where the dependent variables included in the model represent a concept or construct (*e.g.,* "stress response").

Theory of MANOVA. The theoretical or mathematical basis for MANOVAs is matrix algebra (91). In MANOVA, the variance of each DV is accounted for simultaneously by using a matrix of variances and covariances. Similar to ANOVA, the total amount of variance, the variance attributed to the hypothesized model (treatment variance), and the variance due to error is calculated, but unlike ANOVA, where these values are single numbers, in MANOVA each of these values is represented by a matrix. Each of these matrices includes: the sums of squares (represents the total squared differences between the observed value and the mean value) and cross-products (which represents the total values for combined error between two variables). These matrices are used to set up a test statistic (F) which is the ratio of unsystematic or unwanted (error)

variance to systematic variance (variance due to IVs) to determine if differences are significant (91).

Alternate Approach #1: Discriminant Analyses

A discriminant analysis was conducted to examine whether or not the psychobiological variables can predict or discriminate between stress groups and sexes. Discriminant analyses were used to determine if combinations of psychobiological variables can predict group membership for level of stress. Like factor analysis, discriminant analysis identifies a set of underlying dimensions or linear combinations of DVs that best separates the IV groups and can provide a discriminant score based on these relationships (43). The methods of discriminant analysis are similar to, but more powerful than, logistic regression when all assumptions of discriminant analyses are met (43).

Alternate Approach #2: Conceptual Multivariate Models

Three multivariate models were built according to hypothesized relationships that represented each psychological construct, including a: Memory Model, Arousal Model, and Pain Model. Table 3 lists the dependent variables included in each model. These sets of variables were entered into separate MANOVAs to determine if combinations of the psychobiological factors were differentially affected by stress or sex.

Power Analysis

Power analyses were conducted based on the "primary" statistical approach, EFA, followed by MANOVA. The details of the *a priori* power analyses are listed below.

Factor Analysis. Some sources recommend a 10:1 ratio of participants to variables (179), whereas others suggest an $N \geq 50$ with a 5:1 ratio as sufficient (236).

Based on this recommendation, analysis with 16 variables, should have at least 80 subjects. Therefore, with an N = 96, power for the EFA was considered sufficient. Still, interpretation of results may be difficult for small sample sizes (< 150) because correlation coefficients are less reliable (vary from sample to sample) with small sample sizes (187). Small datasets may not generalize as well as those from larger datasets, but might depend on the strength of the inter-relationship between the variables. Generally, if items have high factor loadings (>.80), then the smaller sample size might not pose a problem (236). These criteria were considered when interpreting the results of the EFA.

MANOVA. *A priori* power analyses for MANOVA are complicated because effects sizes are dependent on correlations between dependent variables (Stevens, 1980). Given the DVs in the MANOVA are factors determined using EFA with oblique rotation, there should be a correlation between DVs, and the magnitude of correlations affects the power of the analyses (Stevens, 1980). Despite the complexities of *a priori* power analyses for MANOVA, it is still important to estimate power and the necessary sample size to achieve that power. G*Power 3.16 was used to calculate the sample size necessary. To detect a moderate-large effect (f^2 [V]= .11) with eight groups, four response (DVs) variables, α = .05, at 80% power, a total sample size of 64 subjects is needed. If more than four factors are derived from the EFA, then to detect a moderate-large effect (f^2 [V]= .11) with eight groups, five to seven response (DVs) variables, α = .05, at 80% power, a total sample size of 72 – 80 subjects is needed. Therefore, the ability to detect group differences was considered to be sufficient for ~96 subjects.

Results

The text of the results section presents details about each statistical analysis, rationale for key decisions made relevant to these analyses, and findings revealed by each analysis. This section presents: preparation of data for multivariate analyses; the exploratory factor analysis (EFA); the multivariate analysis of variance (MANOVA); the multiple discriminant analysis (MDA); and three conceptual model MANOVAs. Accompanying tables and figures are cited in the text and appear in Appendices A and B. Additional details related to the analyses are cited in the text and appear in Appendices C and D. Additional summary tables for MANOVA analyses appear in Appendix E.

Preparation of Data for Multivariate Analyses

Multivariate analyses are sensitive to outliers and are based on a number of assumptions (e.g., normality of distribution of values; linearity; 236). Therefore, the dataset was first examined for outliers and distribution of values and all identified problems were resolved before conducting the analyses. Below is a description of how the dataset was prepared for multivariate analyses.

Assessment for missing cases. The dataset of 16 psychobiological variables was first examined for missing values. For 14 variables, there were only 12 missing values/cases out of 1344 total cases (*i.e.,* less than 0.01% missing values). For these cases, values were imputed by taking the mean of the group to which the missing case belonged and using it in place of the missing value.

Two variables (ACTH and Prolactin) each had more than 20 "missing values" out of 96 (> 20%). These values appeared to be missing because the chemist who performed the EIA assays excluded any values markedly outside the range of values expected based

on assay instruction booklet guidelines. The excluded cases did not follow any particular pattern with regard to experimental group. Because imputation is not appropriate with so many missing values, these variables were removed from the analysis (236). Correlations were calculated between these variables and all other variables to determine whether and how these variables were related. Overall, there were a few small, significant correlations (see Table 4). These relationships were considered when interpreting the results of subsequent analyses.

Assessment for normal distribution and univariate outliers. All variables were examined for normal distribution and univariate outliers using methods described in Tabachnick and Fidell (236). Details for each variable with regard to normal distribution and univariate outliers are presented in Table 5. Ten variables had normal distributions without outliers. One variable had a normal distribution, and one outlier was replaced with the mean of the group. The natural log and log base 10 transformations were calculated for five variables because the distributions were skewed, and the mean was not a good indicator of central tendency of the scores in the distribution. For skewed distributions, the median is often a more appropriate measure of central tendency and with transformation the mean and the median become the same (236). The passive avoidance variable used to index memory (PA_Lat) had a significantly negative skew and a limited range (there could be no scores > 300s). The departure from normality was severe for this variable, and no transformation was able to correct it. When transformation is not able to correct a variable, then dichotomizing the variable may improve its utility in analyses (236). For this reason, two new variables were created to represent the memory behavioral variable: one dichotomous and one with four levels.

This variable was adjusted and tested in the factor analysis model (explained below) as a continuous (0-300), dichotomous (cross/did not cross), and finally a quartile (< 100s, 101-200s, 201-299s, 300s) variable.

Assessment for multivariate outliers. The dataset (including 14 variables; *i.e.,* excluding ACTH and prolactin [PRO]) was then screened for multivariate outliers using Mahalanobis distance, derived using SPSS Linear Regression, and evaluated using χ^2 distribution, $p < .001$, $df = 14$ (the number of variables entered in regression), $\chi^2(14) = 36.123$. Mahalanobis distance represents the distance of a case from the centroid of the remaining cases, or the point where means of all variables intersect (236). Cases that fall beyond a certain distance (determined using the χ^2 distribution) may be a multivariate outlier and may need to be removed from the analysis. Based on the criteria listed above, only two values were considered to be multivariate outliers and were replaced by the mean of their respective experimental group. Complete details for this step of data cleaning analyses are presented in Appendix C.

Assessment for linearity. Multivariate analyses also require a linear relationship between variables; non-linear (*e.g.,* curvilinear) relationships are ignored when Pearson's r is used in analyses (*e.g.,* exploratory factor analysis). Linearity is necessary to distinguish among groups of variables and to maximize adjustments for error. Non-linearity reduces the power of the statistical test. The correlation matrix (R matrix) was computed as part of the factor analysis. It was used to examine bivariate linear relationships between variables because *a priori* assessments for linearity (*e.g.,* examination of bivariate scatter plots) included too many possible combinations (14 x 14 = 196) for meaningful visual examination (236).

Exploratory Factor Analysis

The first specific aim was to examine a set of psychobiological variables to identify strongly related variables and to extract the most strongly related linear combinations of these variables using exploratory factor analysis (EFA) with SPSS Version 20 software. An EFA was conducted on the 14 remaining psychobiological variables (see Table 2).

Correlations. The first step of the EFA was to examine the correlation matrix for patterns of relationships among the variables. Variables with no bivariate correlation ($r < 0.3$) and variables too highly correlated (multicolinearity, $r > 0.9$) were considered for exclusion from the analysis. Low bivariate correlation indicates lack of relationship and, therefore, inclusion might weaken the statistical model. Multicolinear variables, or variables with a bivariate correlation of greater than 0.9, are considered to measure an identical construct and inclusion would provide redundant information.

Table 6 presents the correlation matrix for all 14 variables. Some variables had weak bivariate correlations with most other variables (*e.g.,* hot plate[HP], startle [ASR],corticosterone [CORT]; see Table 6), but these variables were retained in the model because there was some relationship among the variables entered into the EFA based on results of Bartlett's test and the value of the determinant (0.039). These measures revealed that there was some relationship between the variables in the model (91). Bartlett's statistic tests the null hypothesis that there is no relationship among the variables. The determinant is a way of describing the "shape" of the data. When there is no relationship among the variables, then the determinant is 1 (91). In this dataset, the determinant was not equal to 1. There was no concern for multicolinearity because all

correlations were < 0.9 and the determinant was > 0 (*i.e.,* the variables were not "too" correlated).

Sample Size. The reliability of EFA is dependent on sample size because correlation coefficients fluctuate from sample to sample especially in small sample sizes (<100; 91). The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was used to determine if the sample size of N = 96 was large enough for the EFA. The overall KMO was 0.583; KMOs of 0.5 to 0.7 are considered mediocre and removal of variables from the EFA model is recommended for variables with KMOs < 0.5 (91). Eight variables had KMO values > 0.5; six variables had KMO values < 0.5. KMOs for all variables are listed in Table 7. Communalities were examined to assist in the decision whether to retain or exclude the six variables with low KMO values.

Communalities represent the proportion of the common variance within a variable. Principal components analysis makes initial assumption that all variance is common. Before extraction, all communalities are = 1. After factor extraction, the communalities represent how much variance the variables actually have in common. Another way to think about communalities is as the proportion of the variance explained by the underlying factors, or the amount of variance in each variable that extracted factors can explain. If there is no shared variance (*i.e.,* no underlying structure or factors), then communalities would be 0. In contrast, variables with no specific variance (*i.e.,* all variance is shared among the variables) have a communality of 1.

Initial communalities for all 14 variables are listed in Table 8. The variables with communalities > 0.6 were considered adequate for a small sample size < 100 (152). Of the 14 variables in the EFA, 13 communalities were > 0.6, meaning for 13 of the 14

variables, 60% of the variance associated with that variable is shared with other variables in the model (91). The only variable with a communality < 0.6 was passive avoidance (0.4). This variable was removed from the EFA model based on the low KMO and low communality. In other words, passive avoidance was excluded from the EFA model because it did not share enough of the underlying variance and did not have enough of a relationship with the other variables to measure underlying constructs. The EFA was then re-run using the remaining 13 variables. All steps and results that follow are based on the 13 variable EFA.

Factor Extraction. To identify the number of factors underlying the EFA model, initial eigenvalues were calculated for each variable entered in the model. Based on the Kaiser criterion, only those factors with eigenvalues > 1 were retained. This analysis yielded six factors with eigenvalues > 1. To determine whether all six factors should be retained and interpreted, two approaches were used.

Based on the Kaiser criterion, all six factors should be retained. For the Kaiser criterion to be considered accurate in sample sizes < 250, there should be: less than 30 variables; communalities after extraction should be > 0.7; and the average communality should be > 0.6 (91). After removing passive avoidance from the model, all communalities were > 0.6 and the average communality was 0.736, but four of the 13 had communalities < 0.7. Therefore, a second approach was used to verify the retention of six factors.

The scree plot (a graph of the factors versus eigenvalues which represent the relative importance of each factor) was then examined (51). Figure 18 presents the scree plot for

this analysis. Based on the point of inflexion (*i.e.,* where the slope changes dramatically), three, four, or five factors should be interpreted.

Checking the model. The next step in the EFA was to examine the reproduced correlation matrix to determine if the relationships among variables were improved by the model (see Table 9). The differences between the original correlations and the model correlations should be small (*i.e.,* the residual should be < 0.05). The EFA that included passive avoidance had approximately 50% of residuals > 0.05, meaning there was a problem in the model. After removing passive avoidance from the model, 44% of the residuals were > 0.05, indicating a slightly better model.

Factor Scores. Factor Scores represent estimates of the score that a subject would receive on each factor had it been measured directly. Factor scores were calculated using the regression approach (91). To improve the estimation of the factor scores, an oblique (rather than orthogonal) rotation was used because it allows factors to correlate. Examination of the component correlation matrix revealed there was little correlation (greatest value was -.174) between any pair of factors, even after oblique rotation.

There were six factors retained from the EFA; details for these factors are presented in Table 10. Briefly, the first factor represents high levels of serotonin activity among the prefrontal cortex (PFC), insula cortex, hippocampus, and basolateral amygdala (BLA). The second factor represents high levels of dopamine activity among the PFC, insula cortex, and hippocampus. The third factor represents low levels of dopamine activity among the PFC, BLA, and central nucleus amygdala (CNA), as indicated by negative factor loadings. The sign of the factor loading is arbitrary, a product of the calculations done in the EFA, and most likely resulted in this analysis because dopamine

activity in the PFC loaded highly onto two factors. However, the underlying meaning of this factor became obvious with the MANOVA (details provide below) and so the direction of the sign was maintained. The interpretation of these negative factors loadings and scores is explained in more detail in the MANOVA section and discussion section below. The fourth factor represents hotplate activity and corticosterone (CORT) levels. The remaining variables fall into the fifth and sixth factors, but may not be interpretable given they do not correlate well with any other variables (*i.e.,* they are what is left over after all other factors are interpreted). Although factors four - six consist of only one or two highly loading variables, all variables and factors were maintained in the EFA model for rotation, to allow for underlying, but weak, relationships to be represented in subsequent analyses. Based on the EFA model and oblique rotation, factor scores for each subject on each variable were estimated (*i.e.,* computed) and were used in subsequent analysis.

Summary of EFA. An exploratory factor analysis was conducted to identify sets of strongly related psychobiological variables. Six factors were revealed. The first three factors appear to represent a relationship in monoamine activity among brain regions. Surprisingly, only one factor (Factor 4) represented a relationship of a behavioral measure (*i.e.,* hot plate) and a biological variable (*i.e.,* CORT). Factors were not retained that represent variables associated with memory, attention/arousal, and pain. The factors identified were used in subsequent analyses.

MANOVA

The second specific aim of this project was to determine if levels of stress (*i.e.,* psychological stress, physical stress, and the combination of psychological and physical stress) and sex (male, female) produced differences in the relationships among the combinations of psychobiological variables identified using the EFA. This aim was addressed using a 4 x 2 multivariate analysis of variance (MANOVA). The first IV was stress with four levels: control, psychological stress ("stress"), injury ("blast"), and psychological stress + injury ("blast + stress"). The second IV was sex with two levels: male and female. The six factors (with factor scores), identified in the EFA, were used as the DVs. (See Appendix E for MANOVA summary tables relevant to this section.)

Cleaning. Because the factor scores estimated from the EFA were new variables, they were checked for normality and outliers. There were no outliers outside 5 SDs and all variables had reasonable skew/kurtosis (they had standardized means = 0 and SD = 1). The next assumption checked was equality of covariance matrices to determine whether variances of each group were roughly equal for each DV, and whether the correlation between DVs was the same for each group. This assumption was checked using Levene's Test. Levene's test was significant for only one variable (Factor 5, $p = .016$), meaning there were unequal variances for the groups for this variable. Because the sample sizes were equal, MANOVA is robust to violation of this assumption (Field, 2009). Box test was used to ensure multivariate normality and the variance-covariance matrices were equal across groups. Box's test was significant ($p < .001$), but because sample sizes were equal, Box test also is robust to the violation of this assumption when Hotelling's or Pillai's statistic are used.

There are several multivariate statistics available to test significance of MANOVA: Wilks' lambda, Hotelling's trace criterion, Pillai's criterion, and Roy's greatest root criterion (grc). Wilks', Hotelling's, and Pillai's are identical when there are only two groups in the analysis (*i.e.,* one degree of freedom). When there are more than two groups, there is more than one way to combine and separate DVs (*i.e.,* dimensions); therefore, these statistics are slightly different. When there is more than one degree of freedom for the effect, then criterion of Wilks', Hotelling's, and Pillai's pool the statistics from each dimension to test the effect (236), whereas Roy's grc only tests the effect from the first dimension. Because the Box test was violated in this analysis, Pillai's criterion -- which explains the proportion of the explained variance by the combined DVs (91) -- was used.

Initial Analysis. A 4 x 2 MANOVA with six factors as the DVs (N = 96) was conducted to determine if psychobiological factors were different for different levels of stress, and between males and females. The overall multivariate model for sex was significant, Pillai's Trace = $F(6, 83)=4.78$, $p < .001$, $\eta^2= .257$ (large effect size), 98% power. Examination of the between-subjects effects revealed males and females differed significantly on Factor 1 (M > F, $F[1,88] = 17.53$, $p < .001$, $\eta^2=.17$ [large effect size], 99% power) and Factor 3 (F > M, $F[1,88] = 13.52$, $p < .001$, $\eta^2= .13$[moderate], 95% power). Based on these results, it appears for Factor 1 (which represents high levels of serotonin activity in the PFC, insula cortex [IC], hippocampus [HIP], and BLA), males had positive factor scores representing greater serotonin activity among the four brain regions, whereas females had negative factor scores representing lower serotonin activity in these brain regions (see Figure 19). Additionally, on Factor 3 (which represents low

levels of dopamine activity in the PFC, BLA, and CNA), females had positive factor scores representing less dopamine activity among these three brain regions, whereas males had negative factor scores indicating greater dopamine activity in these areas (see Figure 20). Though the directions of these factors seem to be opposite from the MANOVA, males had greater levels of monoamine activity in these brain regions, whereas females had lower levels of monoamine activity in these areas.

The overall multivariate model for condition and the sex x condition interaction were not significant ($p > .1$), and there were no significant between-subjects effects for either variable or interaction. Therefore, there was no apparent difference among stress conditions for any of the factors.

Next, a 4 x 2 MANOVA with five factors was conducted to determine if the sixth factor was important for the overall model. There was no change to the significant/non-significant effects. Because there was no difference between the five- and six-factor MANOVA models, there was no legitimate reason to drop the sixth factor. Therefore, all six factors were used in subsequent internal analyses.

Internal Analyses. Because there was a significant main effect of sex, the dataset was then split by sex, and a MANOVA for stress condition was run on six factors as DVs ($N = 48$ for each sex). A MANOVA was conducted to control for Type I error. After splitting by sex, Box's test was no longer significant for either sex; therefore, multivariate normality was assumed. Levene's test was significant for Females, Factor 5, but sample sizes were equal, so the test should be robust to violation of this assumption. The overall multivariate model for condition was not significant for either sex ($p > .1$), and there were no significant between-subjects effects for either sex. It should be noted

that pairwise examination within each sex for simple effects revealed that in the model (*i.e.,* not outside of the model) for Males, Blasted rats had significantly (p = .042) higher loading (.405) on Factor 4 (CORT/Hot Plate [HP]) than did stressed rats (-.393). This finding might be a Type I error, but it is interesting to consider that blast alone stands out from stress alone on this factor for male rats. Hot plate latencies and CORT levels increased together (*i.e.,* more corticosterone was associated with less pain) and Male, Blasted rats had higher factor scores on this factor than Male, Stressed rats (see Figure 21).

Because the factors were not correlated with each other, separate 4 x 2 ANOVAs were run on each factor. There were no additional significant findings.

Summary of MANOVA. A 4 (control, stress, blast, blast + stress) x 2 (male, female) MANOVA was conducted on the factors identified using EFA to determine if there were differences based on level of stress and/or differences for males and females. This analysis revealed *males and females were significantly different* on these factors, especially *with regard to:* (1) serotonin activity among the PFC, IC, HIP, and BLA, where males had positive factor scores and females had negative factor scores; and (2) dopamine activity among the PFC, BLA, and CNA, where females had positive factor scores and males had negative factor scores. Surprisingly, no apparent differences among stress levels on these factors were revealed using MANOVA.

Multiple Discriminant Analysis

Multiple discriminant analysis (MDA) was used in this project as the first, alternate statistical approach to determine if combinations of psychobiological variables could be used to differentiate among levels of stress (control, stress, blast, blast + stress) and between males and females. Discriminant analyses can be used to interpret patterns of differences among the psychobiological variables as a whole to understand the dimensions along which groups differ and to predict membership in a particular group (236).

Initial Analysis. MDA was performed using 14 psychobiological variables (see Table 2 for a list of the variables except ACTH and Prolactin) as predictors of membership in eight groups (sex x condition). These variables were all checked for missing values, univariate and multivariate outliers, and normality before conducting factor analysis. Details of this examination are presented in Table 5.

The overall model of seven discriminant functions (based on eight groups) was significant, Wilks' Lambda= .205, $\chi^2(98) = 133.286$, $p = .01$. However, so the model is not over-fit (will not generalize beyond the sample), the total number of predictor variables (*i.e.,* 14) should not exceed the number of cases in the smallest group (n = 12). Therefore, the *test of equality of group means* table and the *pooled within-group correlation matrix* was used to determine which variables should be removed from the analysis based on univariate differences between groups on each variable and the correlation between variables, respectively (97; 182). Three variables were removed based on non-significant, univariate differences between groups and high correlations with other variables in the model, meaning the addition of these variables did not provide

any additional information to the model. The three variables removed were: dopamine in the CNA, dopamine in the hippocampus, and serotonin in the CNA.

The MDA was run again with 11 variables as predictors of eight groups. The Box M statistic was significant (meaning the assumption of multivariate normality was violated), but sample sizes were equal, so MDA should be robust to this violation.

The resulting model of seven discriminant functions using 11 predictor variables was significant, Wilks' Lambda = .275, $\chi^2(77) = 110.520$, $p = .007$. The eigenvalues for each function, the percentage of variance each function accounts for between the groups, and the canonical correlation associated with each function are presented in Table 11. The first function, made up of a combination of serotonin activity in the BLA, IC, and HIP, and dopamine activity in the BLA, accounted for about 47% of the variance between the groups. About 97% of all variance can be accounted for using five discriminant functions. The details for the psychobiological variables that make up each function are presented in Table 12.

The first function (a combination of serotonin activity in the BLA, IC, HIP, and dopamine activity in the BLA) appears to differentiate between males and females. Figure 22 reveals females have negative scores, whereas males have positive scores. Females have low levels of monoamine activity in these brain regions, whereas males have high levels of monoamine activity in these brain regions. The MDA model was not significant when the first function was removed ($p > .350$).

The second function (CORT, Passive Avoidance) appears to separate male rats exposed to blast alone from all other male rats, and separates female rats exposed to stress alone and female rats exposed to blast alone from all other females. These blasted

males, stressed females, and blasted females had higher levels of CORT and were more likely to cross on passive avoidance than all other groups.

The dataset was split by sex because: (1) the first function clearly differentiated between males and females; and (2) the second function revealed different patterns for males and females based on stress condition. An MDA with 11 variables was conducted for each sex. Neither male nor female MDA models were significant (see Table 13 for the results).

Summary of MDA. Multiple discriminant analysis (MDA) was conducted to determine how combinations of psychobiological variables could be used to differentiate between levels of stress (control, stress, blast, blast + stress) and between males and females. Five discriminant functions were identified that accounted for most of the variance in the model, with the first two functions providing the most meaningful distinctions among the groups. The first function revealed *males and females could be differentiated* using serotonin activity in three specific brain regions for which males had greater activity levels than females. The second function revealed, with regard to corticosterone levels and passive avoidance latencies, there was a *different pattern of response to stress condition for males and females*. The MDA complements the factor analysis and MANOVA that were conducted by revealing differential patterns of responses to stress for males and females that may explain why no clear differentiating dimension was identified when the sexes were combined. Additional internal analyses were conducted based on the information provided by the MDA.

Separate MANOVAs

The second alternate approach to determine whether stress affects combinations of psychobiological variables was to examine separate, multivariate models with MANOVAs built based on conceptualized relationships in the dataset.

Memory. To determine if there was a difference among levels of stress and a difference between males and females on six psychobiological variables related to memory (see Table 3; note the behavioral variable was passive avoidance), a 4 x 2 MANOVA was conducted. All variables were tested for missing values, univariate and multivariate outliers, and tested for normality prior to the EFA. The overall memory multivariate model for sex was significant, $F(6,83) = 3.803$, $p = .002$, $\eta^2 = .216$. Because the omnibus MANOVA was significant, investigation into the relationships among IVs and DVs was appropriate (236). Inspection of univariate tests for each DV revealed males and females were significantly different on dopamine activity in the PFC and BLA, where males had greater dopamine activity than did females in these brain regions. Examination of the relationship between the DVs (see Table 14 for these correlations) revealed: (1) dopamine activity in the BLA and CNA correlated 0.659; (2) dopamine activity in the BLA and PFC correlated 0.326; (3) all other variables were weakly correlated (< .3). The significance in the model was driven by dopamine activity in the amygdala and PFC.

The overall multivariate model for stress level was not significant ($p > .9$). The interaction of sex x stress level showed a trend toward significance with $p = .09$.

Attention/Arousal. To determine if there was a difference among levels of stress and a difference between males and females on four psychobiological variables related to

attention/arousal (see Table 3; note the behavioral variable was acoustic startle), a 4 x 2 MANOVA was conducted. The overall attention/arousal multivariate model for sex was significant, $F(4,85) = 5.009$, $p = .001$, $\eta^2 = .191$. Examination of the DVs contributing to the significant effect revealed the same variables included in the Memory model described above were contributing to this model's significance. That is, sex differences in dopamine activity in the amygdala and PFC contributed to this model. The overall multivariate models for stress level and the sex x stress interaction were not significant ($p > .6$).

Pain. To determine if there was a difference among levels of stress and a difference between males and females on six psychobiological variables related to pain (see Table 3; note the behavioral variable was hot plate) a 4 x 2 MANOVA was conducted. The overall pain multivariate model for sex was significant, $F(6,83) = 6.348$, $p < .001$, $\eta^2 = .315$. Because the omnibus MANOVA was significant, investigation into the relationships among IVs and DVs was appropriate (236). Inspection of univariate tests for each DV revealed males and females were significantly different on serotonin activity in the BLA, IC, and HIP, where males had greater serotonin activity in these brain regions than did females.

Summary of Separate MANOVAs. Three multivariate models were built for sets of variables related to memory, attention/arousal, and pain variables. Separate MANOVAs were conducted to examine the effects of stress on the combinations of these variables and to examine differences for males and females. These analyses were conducted because the EFA did not yield the hypothesized results. Examination of these separate, multivariate models revealed *males and females differed* on all three models,

especially with regard to monoamine activity, for which males had greater monoamine activity than females in specific brain regions. There were no differences for these models based on stress condition.

Discussion

This doctoral dissertation project used data gathered from an experimental investigation of psychological and biological effects of blast overpressure (BOP) induced traumatic brain injury (bTBI) and psychological stress in male and female rats and a comprehensive and sophisticated data analytic strategy to examine complex relationships among the behaviors and biology. The data analytic strategy was designed as a series of analyses: (1) to reveal underlying dimensions in the data; (2) to use those dimensions to try to identify the mechanisms of differential effects of four levels of stress; and (3) to determine whether there were sex differences for psychobiological variables.

This research project had two specific aims with three or four hypotheses associated with each specific aim. The discussion addresses this project's findings with regard to each specific aim and hypothesis. Then, these findings are discussed, followed by a discussion of limitations, future directions, summary, and conclusions.

Specific Aim 1

With regard to the first specific aim -- the identification of strongly related psychobiological variables -- the present work used exploratory factor analysis (EFA) and established there were six measureable, underlying dimensions in the data. The dimensions identified seemed to represent **relationships among key brain regions in serotonin or dopamine activity.** That is, the EFA revealed relationships among: (1) serotonin activity of PFC, hippocampus, insula cortex, and basolateral amygdala; (2) dopamine activity of PFC, hippocampus, and insula cortex; and (3) dopamine activity of PFC, basolateral amygdala, and central nucleus amygdala. These findings imply that these brain areas may be communicating with each other to allow them to operate in parallel or in concert. It makes sense that these particular brain areas are communicating

because they are involved in processing and responding to stress (39; 170). The insula cortex is involved in assigning salience to information (65-68; 71). The hippocampus and amygdala contribute information from memory and emotions (60; 138; 167; 170; 171) to this process. The PFC integrates these processes and coordinates responses (7; 8; 109). It is known that serotonin is involved in homeostatic mechanisms (6) and emotional processing (118). The serotonin findings of the present study (in which subjects were exposed to varying levels of stress) may suggest that serotonin activity in these brain areas is involved in psychological processes that occur during stress. Dopamine is involved in arousal and stress responses (69; 118). The dopamine findings of the present study may suggest that dopamine activity in these brains regions also is involved in psychological processes that occur during stress.

This type of analysis and interpretation goes beyond structural examination and addresses function – a concept shared by physiology and psychology dating back to William James' watershed *Principles of Psychology* (1890). Examination of the bivariate correlations produced by the EFA (Table 6) reveals that monoamine activity within each structure (*e.g.,* hippocampus serotonin compared to hippocampus dopamine) is not well correlated for any of the five brain structures. Additionally, for the two areas of the amygdala (BLA, CNA) dopamine activity is highly correlated (r= 0.66), but serotonin activity between these two areas is poorly correlated (r=.25). If the factors produced by the EFA represented "anatomical" rather than "functional" closeness, then the correlations within each brain region would have been higher and they were not.

Neurophysiological and imaging techniques are being used in humans to identify communication among brain regions based on correlation in function (94; 100). The

present findings suggest that animal models could be used to explore correlations among neurochemical functions and conduct true experiments that manipulate conditions to examine possible mechanism that may underlie the operations of functional activity.

With regard to the present doctoral work, it was expected that activity in the hippocampus, the prefrontal cortex, and the amygdala, would relate in some way. This finding was anticipated because these areas are involved in cognitive and emotional regulation (31; 129), are vulnerable to TBI, and also may be associated with PTSD (129; 165; 244). Because many service members are subject to psychological stress and physical stress, such as brain injury, this project attempted to examine the effect of multiple stressors acting simultaneously to elucidate biological mechanisms underlying psychological effects of these different stressors. This doctoral project revealed communication among relevant brain areas, yet differential effects psychological vs. physical stress were not as apparent as hypothesized (see below for a discussion of Specific Aim 2).

An additional dimension represented a **relationship of a behavioral measure of pain** (*i.e.,* hot plate) **and a biological index of stress** (*i.e.,* blood levels of corticosterone). Because higher levels of corticosterone were associated with longer latencies on the hot plate task (indicating decreased nociception or decreased "pain" sensitivity), these findings suggest that corticosterone is anti-nociceptive. It was interesting that the factor analysis yielded a dimension which associated a behavior with a biological marker, but there was no significant difference on this factor between males of females or among stress groups, so the interpretation of what this factor is measuring is limited.

The hypotheses that were associated with Specific Aim 1 (there would be sets of psychobiological variables strongly associated with memory, attention/arousal, and pain) were not confirmed. EFA is an inherently exploratory analysis, and these hypotheses were made based on conceptual and empirical reasoning. Because stress is a complex process that includes psychological and physiological responses, this project attempted to determine whether and how psychological and physiological systems **interact** in response to **simultaneous exposure to more than one** type of stress considering **central physiological** changes, such as neurotransmitters, which may influence psychological function. Stress research throughout the 20th century, which acknowledged the role of psychological responses, stopped short of attempting to identify the underlying or associated physiological responses or systems (84). This project attempted to examine physiological responses, physiological systems underlying psychological concepts (*e.g.,* memory), and behavioral responses to two different stressors.

Unfortunately, the inter-relationships among central, peripheral, and behavioral variables were not as strong or as "simple" as was expected. There are two possibilities to explain why a stronger relationship among these variables was not found. The first and most likely possibility from the author's perspective is that the variables were interacting in a complex way that the statistical analyses were unable to detect. This project attempted to apply a sophisticated statistical approach to examining the complex relationships among psychobiological variables involved in the stress response, but the techniques selected all relied on a linear relationship among the variables (91; 236). These statistical techniques did not allow for curvilinear relationships. This possible limitation is discussed below.

Another possibility why the psychobiological variables were not more strongly related in the present research project may be the behavioral or biological measures lacked sensitivity to assess the psychological constructs. The present project used the Warrior Stress Paradigm (WSP) to induce stress in the rat. Based on univariate analyses from the parent project from which the data for the present project were gathered, the WSP produced differences in behavioral and biological outcomes in response to stress. For example, after exposure to the WSP, male, stressed rats performed worse on measures of neurobehavioral function than did non-stressed males (257; 259); and female, stressed rats had less open field activity than did non-stressed females (257; 259). Additionally, the WSP significantly increased corticosterone in male and female rats compared to non-stressed rats (unpublished data).

The present study also included three behavioral measures that have established validity (see Study of bTBI and Stress, Dependent Variables section in the background of this doctoral dissertation). Perhaps additional measures, which assess the same or similar constructs, or additional time-points of the same measures (*i.e.,* information from baseline and/or subsequent post-experimental manipulation measures) should be included in future experiments to better assess psychological constructs.

Because the Specific Aim 1 hypotheses were not confirmed by the EFA, consistent with the initial project proposal, an alternate analytic approach (three separate MANOVAs) was subsequently conducted. This approach tested three multivariate models built for sets of variables related to memory, attention/arousal, and pain variables. These models were tested for effects of stress on the combinations of these sets of variables and examined differences for males and females. These separate MANOVAs

did not reveal any additional information beyond the results of the primary MANOVA conducted on factor scores derived from the EFA. This alternate approach did, however, reveal sex differences that were consistent with the MANOVA and multiple discriminant analysis conducted to address Specific Aim 2.

Specific Aim 2

With regard to the second specific aim -- testing for differences among stress groups and between males and females for combinations of psychobiological variables -- the present work revealed males had greater levels of monoamine activity in specific brain regions than females, specifically: **(1) males had positive scores on the factor representing higher levels of serotonin activity among specific brain regions and females had negative scores on this factor, representing lower levels of serotonin activity in these areas;** and **(2) females had positive scores on the factor representing lower levels of dopamine activity among other specific brain regions whereas males had negative scores on this factor, representing higher levels of dopamine activity in these regions**. The present project also revealed males and females showed different patterns of response to stress, but no significant effects of stress were identified. The first three hypotheses that were associated with Specific Aim 2 (the three psychobiological constructs of memory, attention/arousal, and pain, would be differentiated by stress condition) were not confirmed because there were no significant differences among stress groups. **The fourth hypothesis (combinations of psychobiological variables would be different for males and females) was partially confirmed.** Males and females appeared to have differences in neurochemical function, but a clear difference based on sex *and* stress condition was not found.

Sex differences in brain function using measures of functional connectivity among brain areas has been reported for humans based on functional neuroimaging (100; 124; 241). However, it has not been established if these differences reflect "better" or "worse" responses and outcomes. The fact that the present study revealed sex differences in neurochemical function of major monoamines among brain regions relevant to psychological processing, suggests that animal models and the quantitative analyses used in this project might be used to explore sex differences in brain function in more detail.

Sex differences in brain function are just beginning to be identified. Although "how" males and females function differently is becoming clearer, "why" males and females function differently is not at all clear and has not yet been addressed. True experiments using animal models of neurochemical function may be able to examine this "why" question. For example, does differential neurochemical function in male and female brains reflect some aspect of survival that has "outlived" its usefulness? Or, does differential neurochemical function in male and female brains have some useful purpose that should be studied in order to try to improve health of males and females in a modern world? Taylor et al (240) offered a "female" stress response that is presumed to operate along with the classical fight-or-flight "male" response. The present findings of sex differences in neurochemical function suggest that there is a lot more going on in the brain that differentiates males and females.

Additionally, because differences in brain function are evident, using measures of functional connectivity, in pathological states, such as migraine (146) and panic disorder (102), preclinical investigations using animal models may be used to test novel interventions or therapeutics that differ for males and females based on differential

neurophysiological function. For example, it has been speculated that bupropion, an antidepressant used to aid smoking cessation which inhibits the reuptake of dopamine and norepinephrine, is less effective for helping males to quit smoking than for helping females to quit smoking, even though it is more difficult for females to quit smoking (214). The exact mechanisms for these sex differences are not known. It is possible that differences in monoamine activity (*i.e.,* serotonin, dopamine) among different brain regions for males and females may explain the differential effectiveness of this drug. Future projects using animal models to measure neurophysiology and multivariate statistical analyses to assess for relationships among brain regions based on function could further examine these sex differences in effectiveness of medications.

In the present project, MANOVA was used as the primary approach to determine how males and females were different and if there were any difference among stress condition for the psychobiological factors derived using EFA. This analysis revealed differences based on sex but not stress. Multiple discriminant analyses (MDA) was used as a second approach to answer questions about sex and stress differences among the psychobiological variables. MDA, like factor analysis, was used to identify sets of underlying dimensions that best separate males and females and stress groups (43). Consistent with the MANOVA, the first function revealed by the MDA was *males and females could be differentiated* using serotonin activity in three specific brain regions for which males had greater activity levels than females.

Examination of the data from the present project revealed males and females showed different patterns of response to stress, but no significant effects of stress were identified. The interaction of sex and stress showed a trend toward significance, but

given the *relationships* among psychobiological variables appeared to vary from bivariate combination to bivariate combination and no consistent patterns emerged, there may not have been enough power to detect subtle differences among stress groups between or within sex. The issue of power is discussed below in the limitations section and more information about the different patterns of relationships among variables is presented in Appendix D.

Limitations

Sample size. Multivariate analyses allow for examination of more than one variable simultaneously which can take into account relationships among variables that may affect outcome. Effects of variables on each other can be missed simply by using univariate analyses. The present research project used several multivariate analytic techniques (EFA, MANOVAs, and MDA) to analyze empirical data gathered from an experimental investigation of psychological and biological effects of stress. The statistical approach used in the present study is commonly used in studies conducted with large numbers of human participants, but has rarely been applied to preclinical experiments with animal subjects. The rationale for the present study was to use multivariate analytic techniques with psychological and biological variables that are likely related to each other in order to reveal relationships and patterns that cannot be determined by univariate analyses alone.

Multivariate analyses usually are performed on large sample sizes of humans who have varied demographics and life experiences. In contrast, laboratory experiments use relatively small samples (< 100) because error variance of individual responses is minimized in true experiments that control for subject age, genetics, prior experience,

housing, exposure to independent variables, and so on. The present project was based on an animal experiment that used 96 subjects and measured many psychological and biological variables. It is noteworthy, however, that sample sizes < 100 often are considered to be too small to detect effects in multivariate models (91; 236).

Despite its relatively small sample size, results from this project were interpretable, and it can be added to the list of empirical investigations using multivariate models in small samples that yield results. However, the sample size of 96 must be considered to be a study limitation that would likely be addressed by using a larger sample size. Based on the effect size of the current sample (Pillia's Trace = .164, with 59% power), to detect differences (with 80% power) among stress groups when males and females are combined would require 176 subjects (a reasonable number for future animal experiments). Based on the effects size of the current sample (females [N = 48]: Pillia's Trace = .395, with 48% power; and males [Pillia's trace = .281, with 69% power), to detect stress differences (with 80% power) when the data are split by sex, 52 females and 72 males would be required (quite reasonable numbers for future animal experiments). Therefore, future preclinical experiments using male and female rats that will be analyzed using multivariate analyses should include ~150 subjects (75 male, 75 female).

Linearity of relationships. Linearity is an assumption of all multivariate tests used in this project (i.e., all variables entered into each model must share a linear relationship; 236). It is possible, given the complex ways that psychobiological variables may be interacting, that relationships among these variables are not linear. For example, the relationship between arousal (or stress) and various psychological outcomes often

follow an inverted U-shaped relationship (105; 261). It also is possible that the variables relate in different ways based on stress condition for males *vs.* females (see Appendix D for a discussion of these differential correlations based on sex). Other techniques, such as neural network modeling and support vector machine analyses which do not rely on linearity (56), may be useful approaches to study psychobiological responses to stress. However, these analytic techniques also are limited in small samples, so it remains an empirical question whether they would work.

Timing of data collection. In the parent project from which the data for this project were drawn, biological variables were collected days after behavioral variables because currently available techniques for collection of biological samples (*e.g.,* blood sampling and brain tissue collection) are invasive and would influence experimental outcomes. It is possible that stronger associations between the behavioral and biological variables were not revealed because of this timing of behavioral and biological measurements were not coincident. If the variables had been measured simultaneously, then stronger relationships may have been found. The approaches and timings used in the parent project to collect psychological and biological variables are the best approximations currently available.

Future Directions

Based on the findings of the present study and the limitations listed above, there are a number of possible future directions.

Sample size. Multivariate statistical techniques can and should be used to examine datasets that include psychological and biological variables that may relate in complex ways. If possible, sample sizes > 100 should be used if multivariate statistical

techniques are planned and *a priori* power analyses should be conducted based on expected effects of the experimental manipulations.

Linearity of relationships. If linear multivariate analyses do not yield clear findings, then it would be wise to try non-linear multivariate techniques such as neural network modeling and support vector machine or learning machine analyses (56). The data analyzed in the present project could be analyzed in these ways: (1) to compare to linear techniques used in this project, and (2) to determine if these statistical techniques can be used effectively in samples < 100.

Timing of data collection. To try to better match the collection timing of behavioral and biological measures, biological sample collection techniques, such as brain imaging and blood collection via indwelling catheters, could be used. These approaches, however, can be expensive, require substantial technical expertise and sophisticated equipment, and still may affect the outcome (especially when stress is a variable of interest).

Further experiments. Based on the present findings, it would be useful to encourage all investigations of stress (human and animal) to include sufficient numbers of male and female subjects to allow for meaningful consideration of main effects for sex as well as interactions of sex and stress. Listed below are additional guidelines and suggestions for future experiments in animals and humans that may extend the findings of this project.

Animal experiments. There are many ways to extend the findings of the present project for use in future experiments with animals by altering the independent and dependent variables manipulated and measured.

The current project attempted to distinguish between physical and psychological stressors to provide information to the differential diagnosis of TBI vs. PTSD, but was unable to statistically distinguish between the levels of stress. However, this project found strong difference in psychobiological responses for males vs. females. To extend the findings of this project and to examine sex differences in stress responses, future investigations may benefit by simplifying the stress condition to a "stress vs. no stress" independent variable. To model stressors Warriors experience, this stressor should represent a compound physical and psychological stressor.

The stressors used in this project were mild physical and psychological stressors. They could be altered to represent more severe exposures. For example, the blast could be altered to model exposure to multiple mild blast injuries. Reports from the field indicate Warriors are commonly exposed to multiple blasts (especially from IEDs) and multiple blast exposures may be particularly harmful (127; 204; 208). Future experiments should combine multiple blast exposures with the Warrior Stress Paradigm and behavioral and biological measures to detect effects on psychological and neurochemical variables.

Another way to alter the stressors would be to include sleep disruption or deprivation in combination with the blast exposure(s). Deployed Warriors are subject to sleep disruption and deprivation that affect their performance and behavior. Perry (188) used an animal model to study the deleterious effects of sleep disruption on behavior. This stressor could be added to the blast injury to provide a more complete model of the Warrior's experience of stress that occurs in addition to the brain injury.

Additional dependent variables could be added to extend the findings of this work. This project was part of a larger experimental investigation of stress and blast injury for which there is additional behavioral and biological data. Additional behaviors include indices of anxiety-like (center time in an openfield) and depression-like (vertical activity in an openfield) behaviors as well as neurobehavioral functioning (Neurobehavioral Severity Scale-Revised version [NSS-R]; 259). Additional biological variables include measures of peripheral immune responses from plasma and serum levels of chemokines and cytokines.

This project was part of the Center for Neuroscience and Regenerative Medicine (CNRM), a collaboration between the Uniformed Services University (USU) and the National Institutes of Health (NIH). Projects in the CNRM were designed and organized to be somewhat methodologically similar to allow for "pooling" of the data collected. A future direction to extend the findings of this study and increase the sample size may be to use behavioral and biological data collect from other CNRM studies using rats to study brain injury and stress in male and female rats.

Another way to improve and extend the findings of this project may be to include pharmacologic manipulations of neurochemical activity to "prevent" or "treat" responses to stress. The present project found differences in monoamine activity in the brains of male and female rats. Drugs that manipulate levels of monoamines in the brain (*e.g.,* selective serotonin reuptake inhibitors [SSRIs], dopamine-norepinephrine reuptake inhibitors [DNRIs]) could be used to further elucidate the behavioral and biological outcomes of differential monoamine activity in the brain.

Human studies. Based on the findings of the present project, it would be useful to follow up by conducting studies that examine differences in neurochemical function as related to behavior and cognition in males and females exposed to different types and levels of stress. More specifically, it would be interesting to conduct studies with human subjects who have TBIs and who have been diagnosed with or without stress-related disorders, such as post-traumatic stress disorder (PTSD). If the present findings generalize to the human situation, then there should be sex differences in neurochemical function related to dopaminergic and serotonergic activity that might be detectable using functional neuroimaging (*e.g.,* positron emission tomography [PET]). Future examination of sex differences in brain function in humans using neuroimaging, similar to the preclinical experiments suggested above, may provide information into "how" and "why" and "what-to-do-about" differential neurophysiological function between males and females.

In addition, based on the literature reviewed in the background section of this doctoral dissertation, it would be worthwhile to examine whether there are stress or sex x stress effects on brain function in human samples with and without TBI and/or PTSD. If these potential future experiments revealed differences in neurochemical function based on sex and/or stress, then that might suggest using different pharmacological interventions (or other approaches that would manipulate brain chemistry) in males and females, to treat these conditions.

Summary

The purpose of this doctoral dissertation project was to characterize psychological and biological responses in a rodent model of Warrior Stress using males and female rats. A comprehensive and sophisticated multivariate data analytic strategy examined complex relationships among behavioral and biological responses to physical and psychological stress. Data were gathered from an experimental investigation of blast overpressure induced traumatic brain injury and psychological stress in male and female rats. Exploratory factor analysis, multivariate analysis of variance, and multiple discriminant analysis were performed: (1) to reveal underlying dimensions in the data; (2) to use those dimensions to try to identify the mechanisms of differential effects of four levels of stress; and (3) to determine whether there were sex differences for psychobiological variables.

Multivariate analyses revealed **males and females differed on dimensions that represented brain monoamine activity, specifically**: (1) serotonin among the prefrontal cortex, hippocampus, insula cortex, and basolateral amygdala, and (2) dopamine activity among the prefrontal cortex and the amygdala. There were no apparent differences among stress groups on combinations of the psychobiological variables. This project highlights the need for considering sex differences in psychobiological responses and for including males *and* female participants in all injury and stress research.

Conclusion

The findings of this project contribute to the growing literature that highlights psychological and biological differences between males and females. This project started as an attempt to determine how different types or levels of stress -- physical stress and psychological stress and their combination -- contribute to the development of post-deployment related symptoms including memory problems, concentration problems, and pain in attempts to improve the diagnosis and treatment of TBI and PTSD. Several multivariate statistics -- which are typically applied to large scale, human studies -- were tested using a small (*i.e.,* < 100 subjects) dataset from a preclinical investigation of psychological and biological effects of stress. Multivariate statistics were used in this "less-than-conventional" way because, based on the stress literature, the psychobiological variables included in the present project were expected to relate in complex ways. These techniques were used to dig into the dataset and extract sets of complex relationships that were not apparent based on observation, univariate, or even bivariate analyses. In translational and/or interdisciplinary research, which includes investigations into behavior and biology, it is rare for researchers to statistically or quantitatively combine the different dependent variables beyond simple bivariate correlations.

The results of the project, which reveal **sex difference in physiological mechanisms in the brain**, demonstrate that these analytic techniques can be employed in a small sample when the magnitude of the effect is large. Because differences based on stress group were not identified using these techniques, it is possible that the effects of stress are more subtle than the effects of sex or are non-linear and, therefore, may require more cases to be examined and/or use of a different statistical analysis (e.g., neural

network modeling). Based on this assessment, for future instances when multivariate techniques seem to be the appropriate statistical approach to address the research question, sample sizes should be at least 100 based on the size of the effect expected.

Use of these multivariate analyses has greatly expanded my statistical analytic knowledge and abilities. This experience digging into the empirical literature on topics from many different perspectives (including medical psychology, neuroscience, clinical psychology, medicine, pharmacology) and across these disciplines to conceptualize psychobiological models worth testing has been extremely valuable. Additionally, this work has helped me gain insight regarding the selection of statistical approaches, how to compare and contrast various analytic approaches, and to deeply appreciate the utility of a broad range of statistical techniques. The knowledge gained performing this research project in addition to the years of training as prelude to this project has prepared me well for a career as a U.S. Army Research Psychologist with expertise in psychobiology and quantitative methods.

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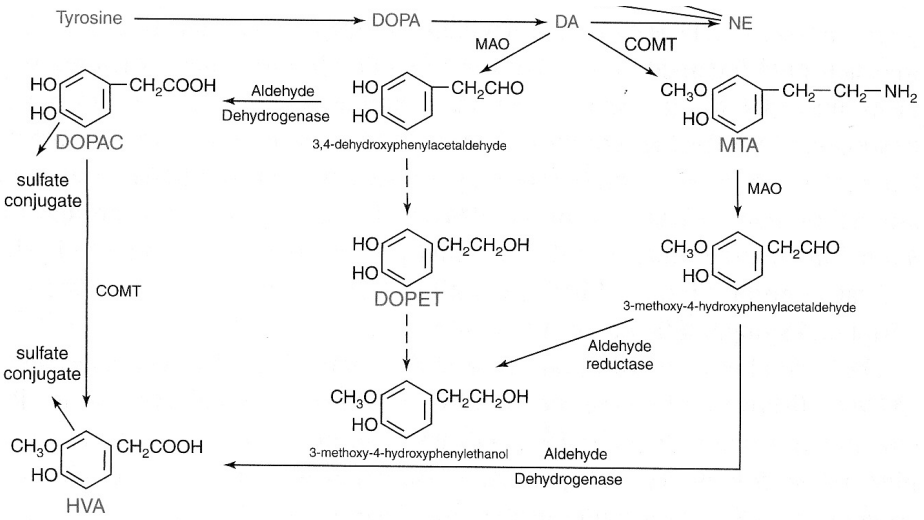
Appendix A: Figures

Figure 1

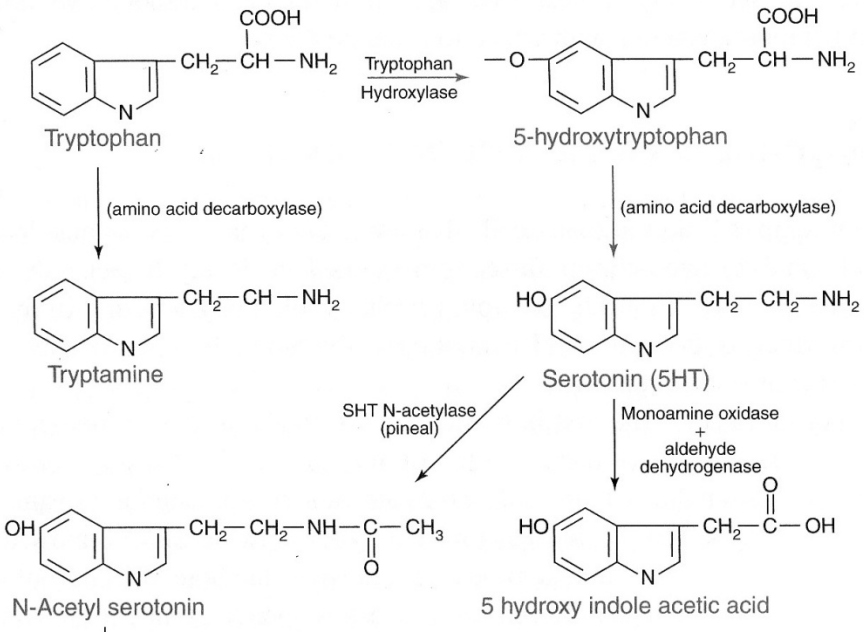
Dopamine Metabolic Pathway



(from Iversen et al., 2009, p. 163)

Figure 2

Serotonin Metabolic Pathway



(from Iversen et al., 2009, p. 216)

Figure 3

Stress Equipment

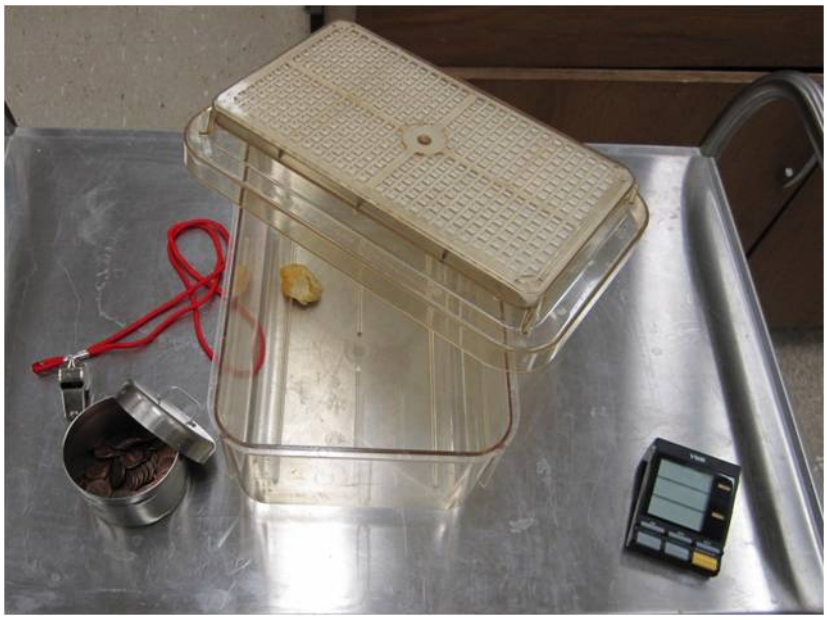


Photo by A.M. Yarnell

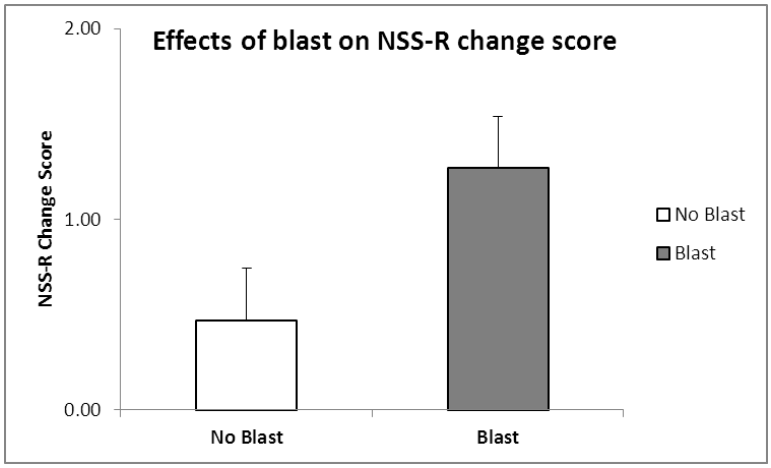
Figure 4

Warrior Stress Paradigm Timeline

DAY 1	PREDATOR STRESS 20 MIN
DAY 2	PREDATOR STRESS (REMOVE AFTER 10 MIN) THEN WHISTLE @ 12, 15, AND 19 MIN
DAY 3	PREDATOR STRESS (REMOVE AFTER 10 MIN) THEN COIN SHAKE @ 11, 14, AND 17 MIN
DAY 4	PREDATOR STRESS (REMOVE AFTER 10 MIN) THEN FLASHING LIGHTS @ 13, 16, 18, AND 19 MIN
DAY 5	PREDATOR STRESS (REMOVE AFTER 10 MIN) THEN CAGE SHAKE @ 12, 15, AND 18 MIN
DAY 6	PREDATOR STRESS (REMOVE AFTER 10 MIN) THEN FLASHING LIGHTS @ 12, 16, AND 19 MIN
DAY 7	PREDATOR STRESS (REMOVE AFTER 10 MIN) THEN WHISTLE @ 11, 13, 16, AND 18 MIN

Figure 5

Impaired Neurobehavioral Function following blast exposure



Yarnell, 2012

Figure 6

Metal, Mesh Basket

Yarnell et al., 2013

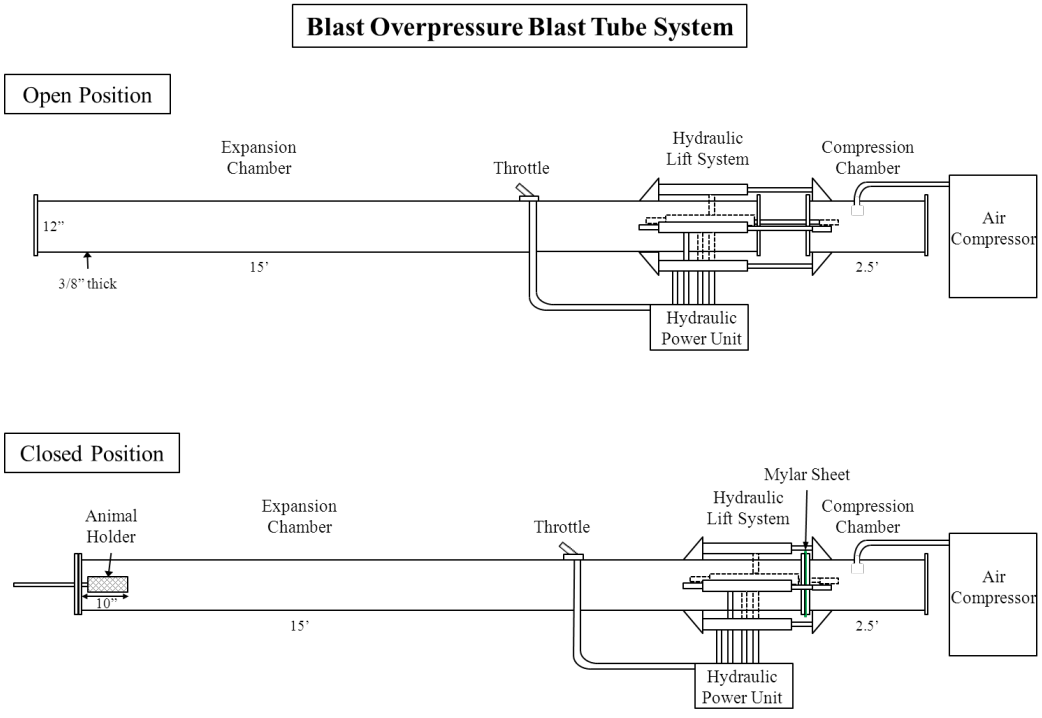
Figure 7

Blast Tube Opening

Yarnell et al., 2013

Figure 8

Blast Tube Schematic



Yarnell et al., 2013

Figure 9

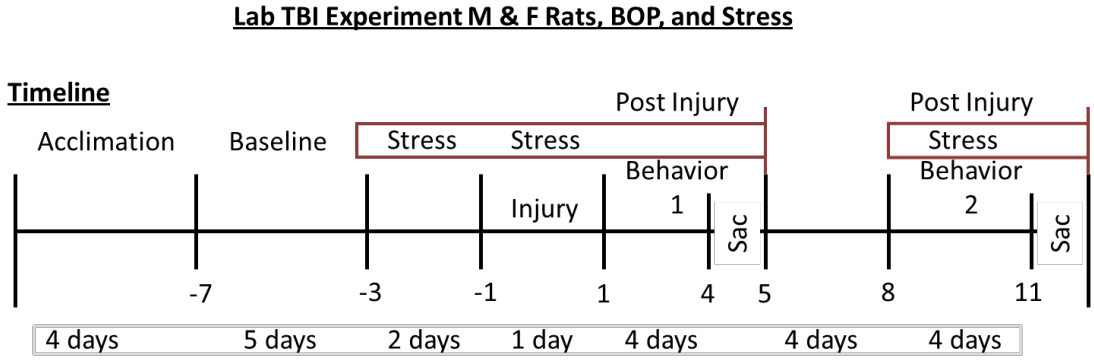


Figure 10

Passive Avoidance Equipment

Photo by A.M. Yarnell

Figure 11

Acoustic Startle Reflex (ASR) Equipment

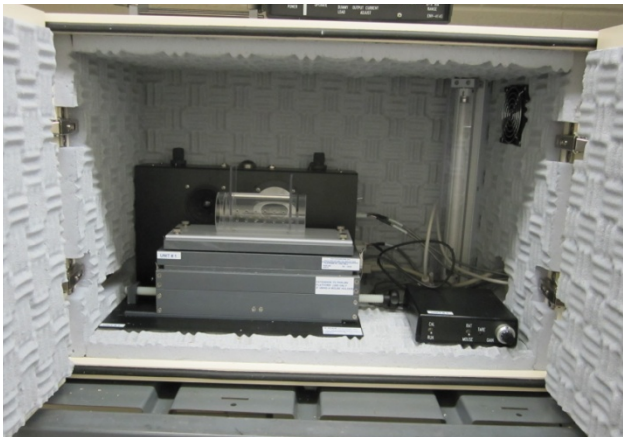


Photo by A.M. Yarnell

Figure 12

Hot Plate Equipment

Photo by A.M. Yarnell

Figure 13

Jacobowitz Brain Block

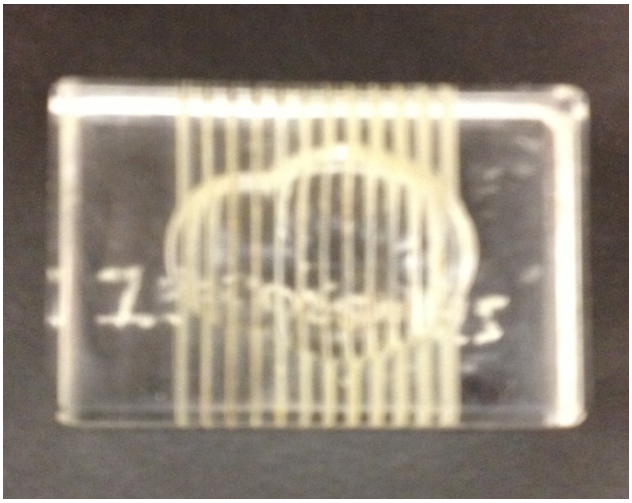


Photo by A.M. Yarnell

Figure 14

Brain Slices on Slide

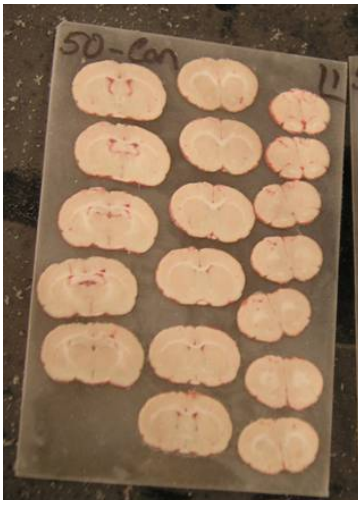


Photo by A.K. Starosciak

Figure 15

Psychobiological Variables Associated with Memory

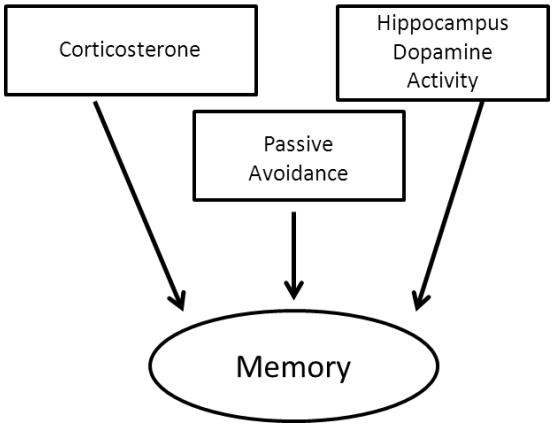


Figure 16

Psychobiological Variables Associated with Attention/Arousal

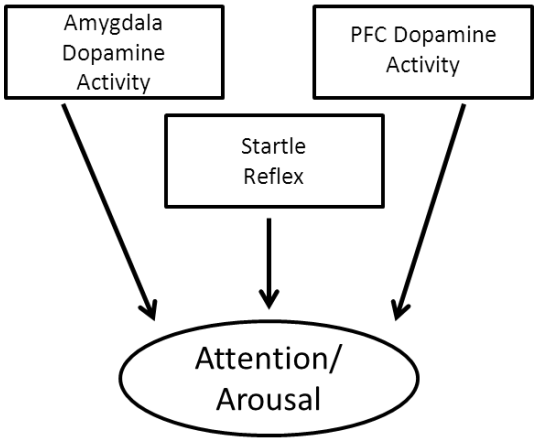


Figure 17

Psychobiological Variables Associated with Pain

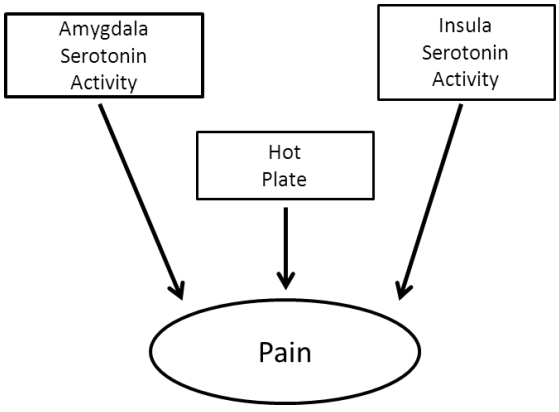


Figure 18 **Scree Plot for Exploratory Factor Analysis**

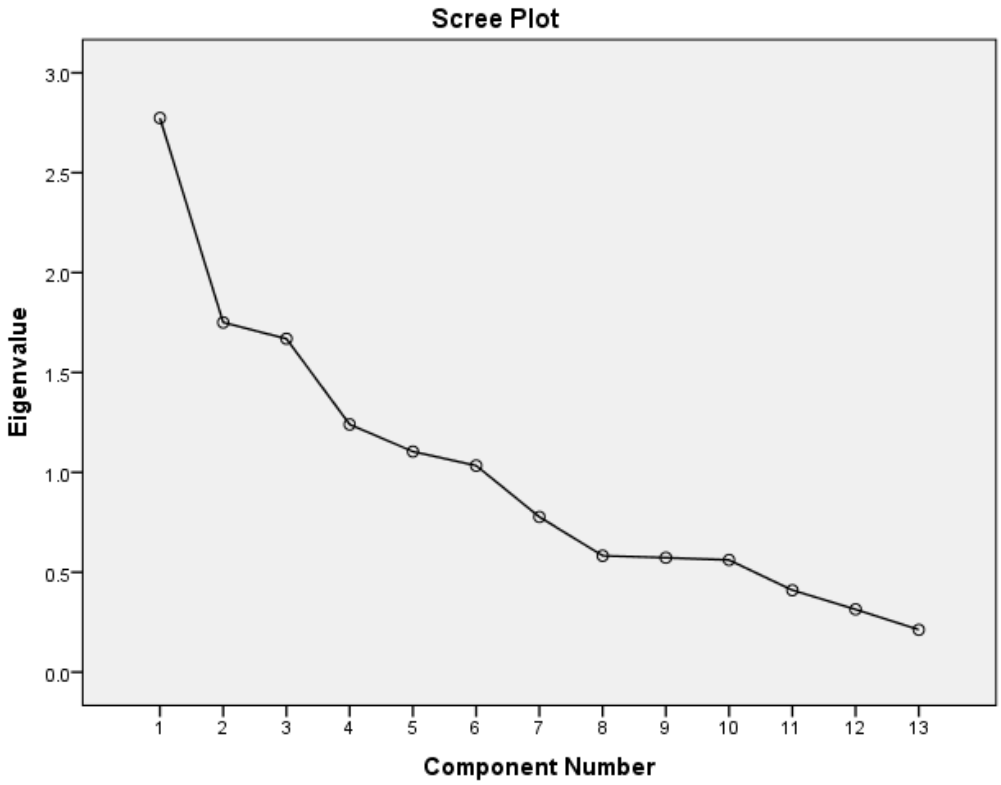


Figure 19

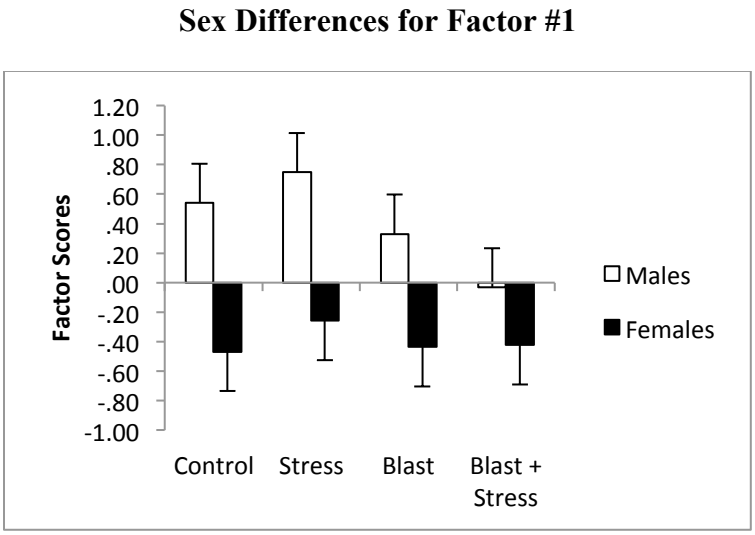


Figure 20

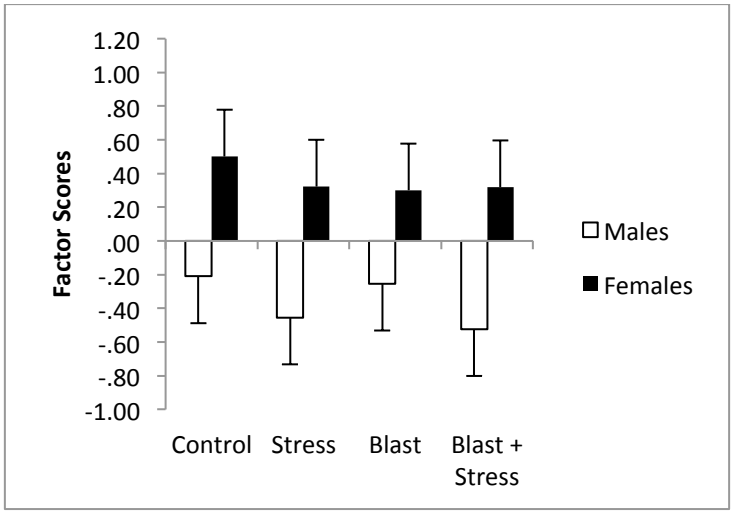


Figure 21

Effects of Stress in Male rats for Factor 4

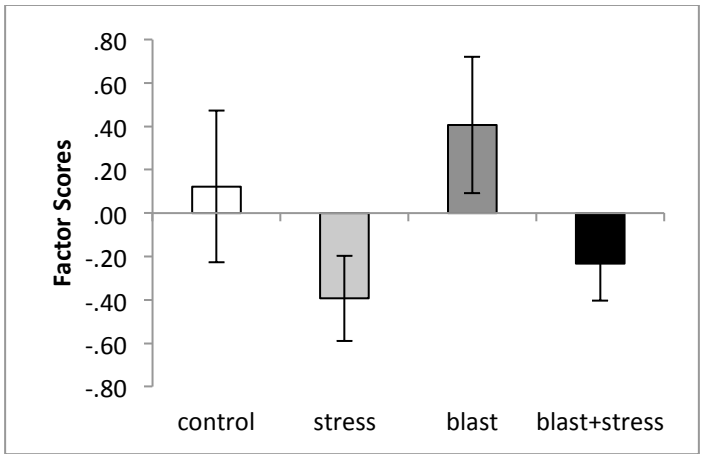


Figure 22 **Sex differences for Function #1**

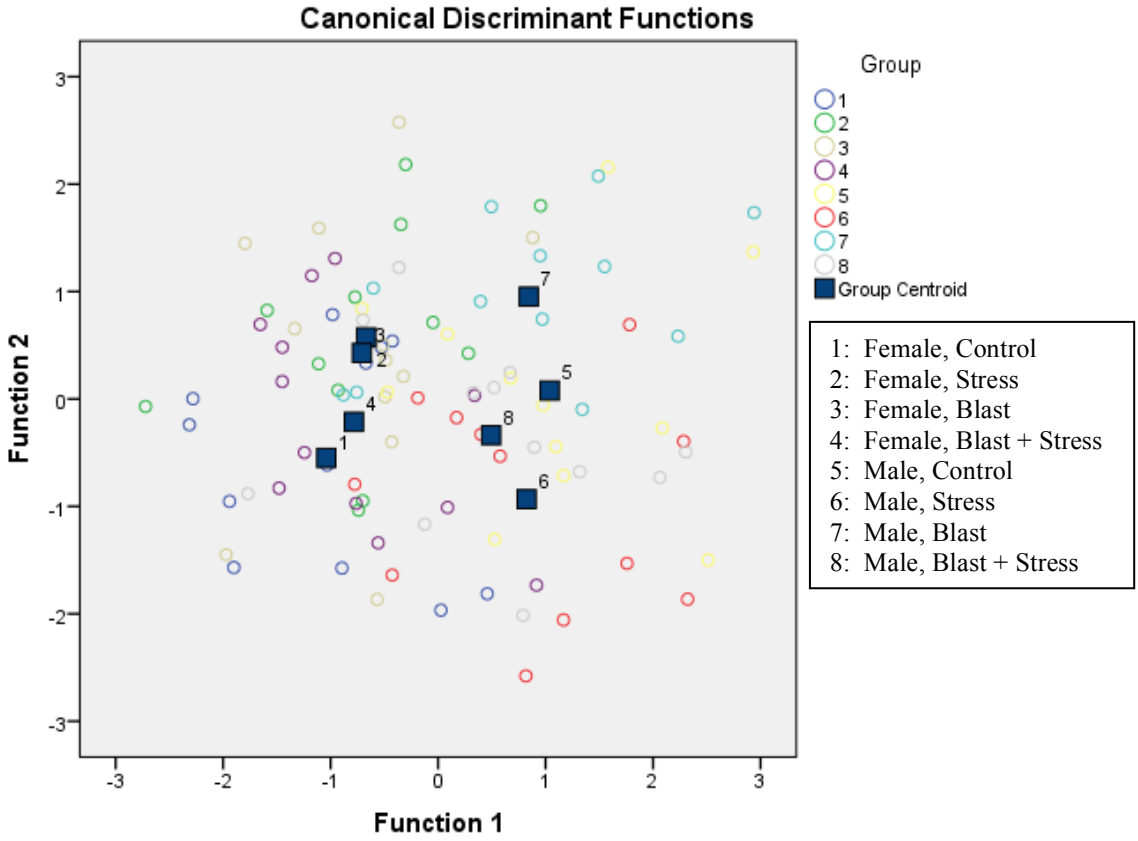


Figure 23 Factor 1 vs Startle

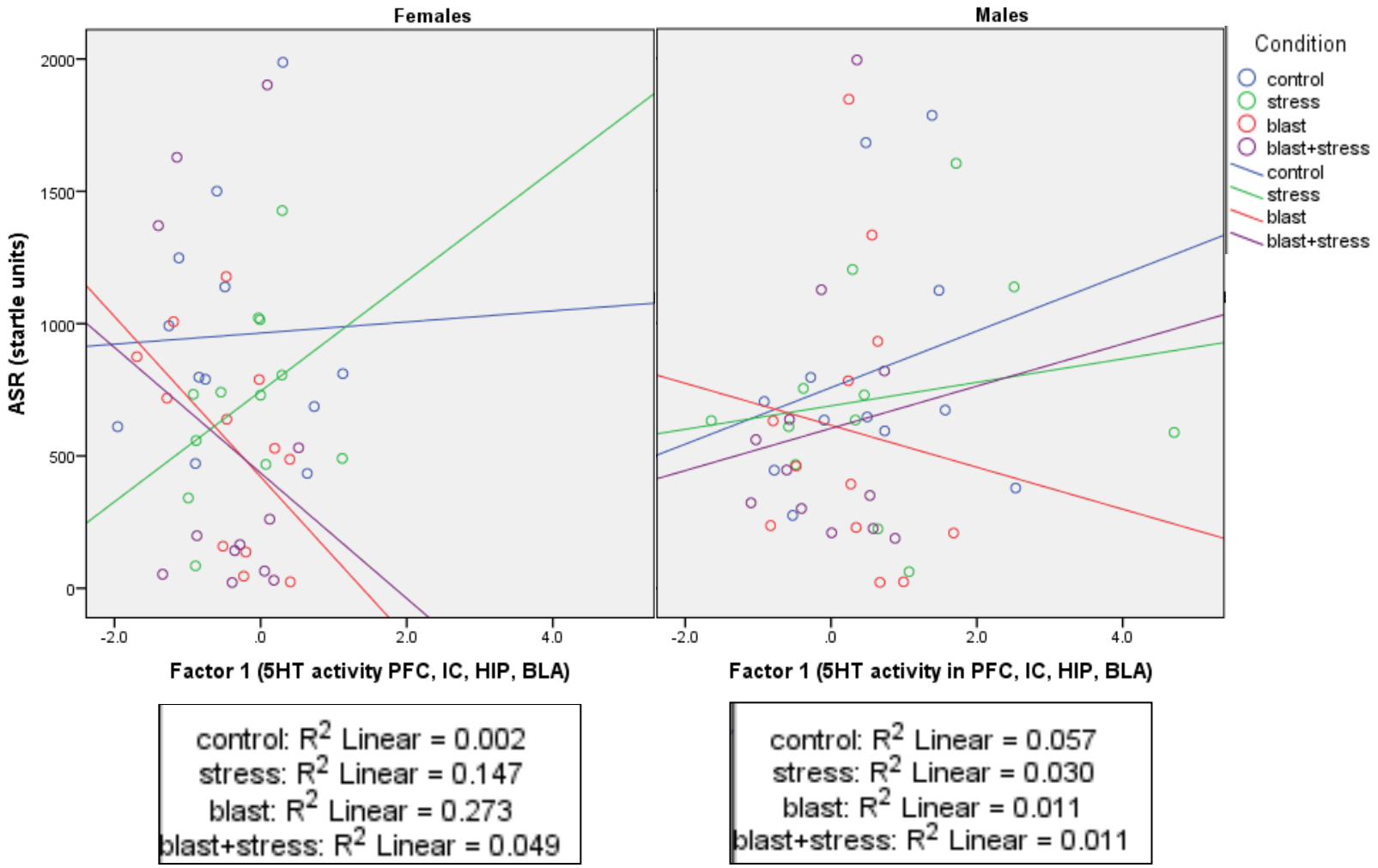


Figure 24 Factor 2 vs Startle

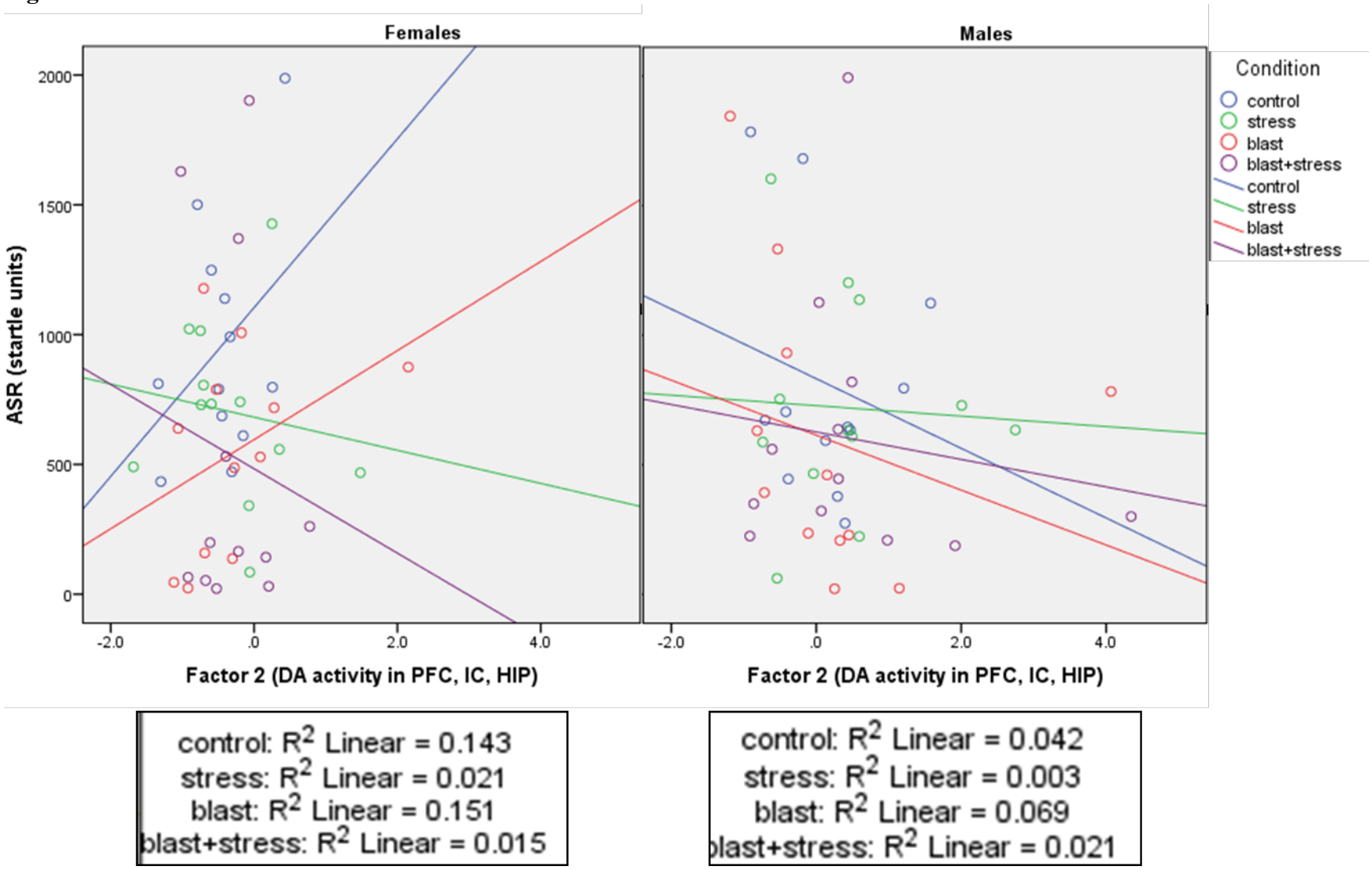


Figure 25 Factor 3 vs Startle

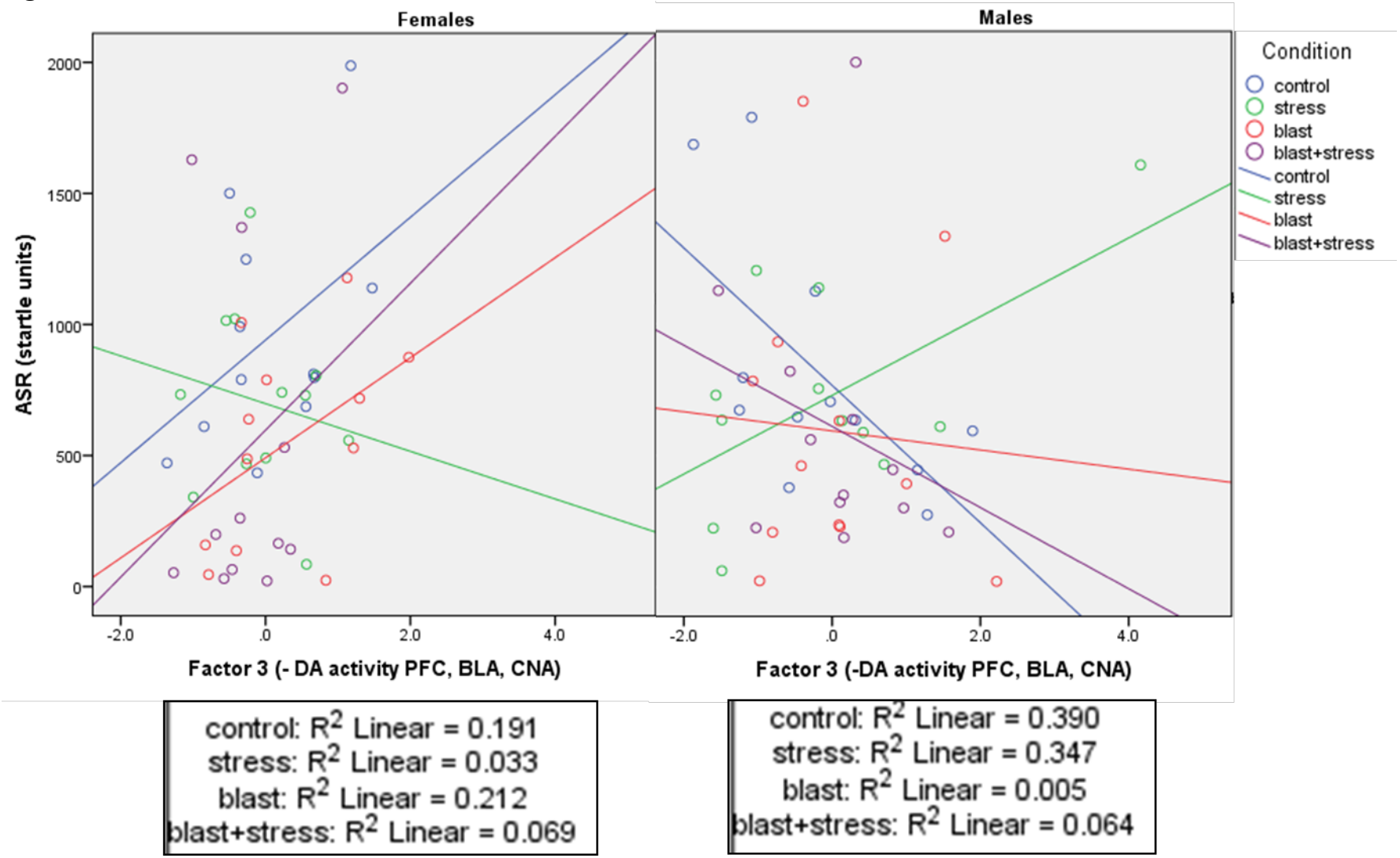


Figure 26 Factor 1 vs Hot Plate

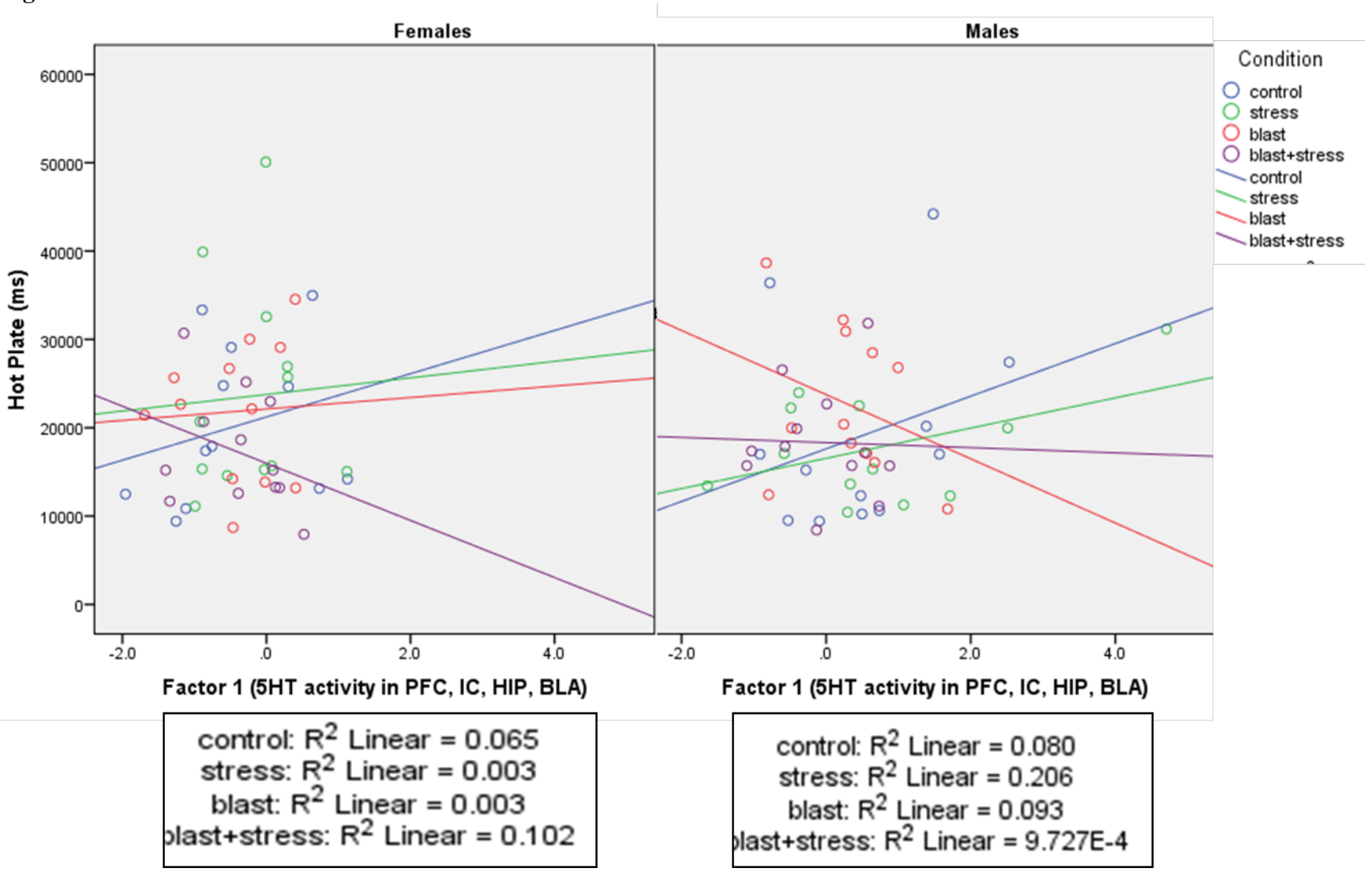


Figure 27 Factor 2 vs Hot Plate

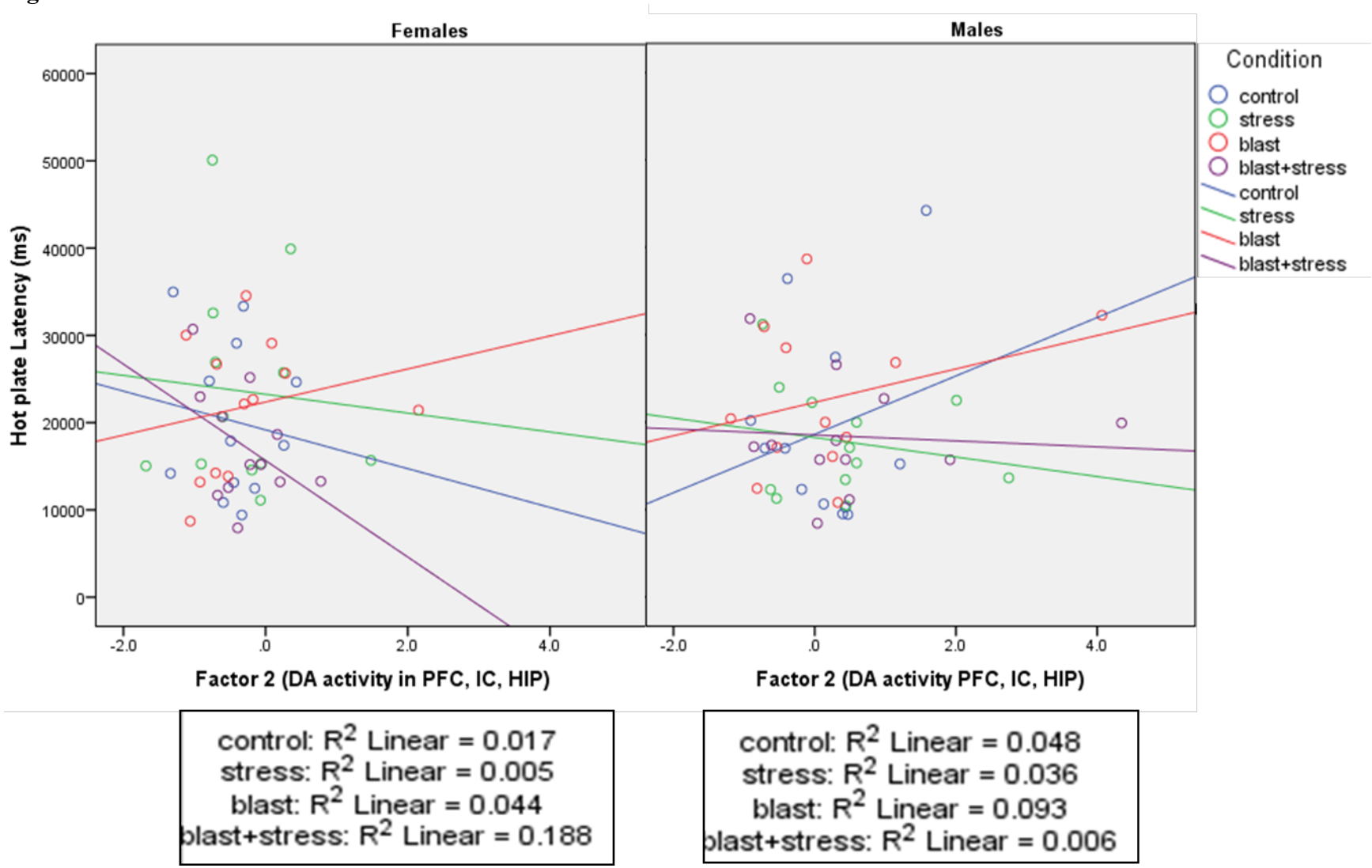
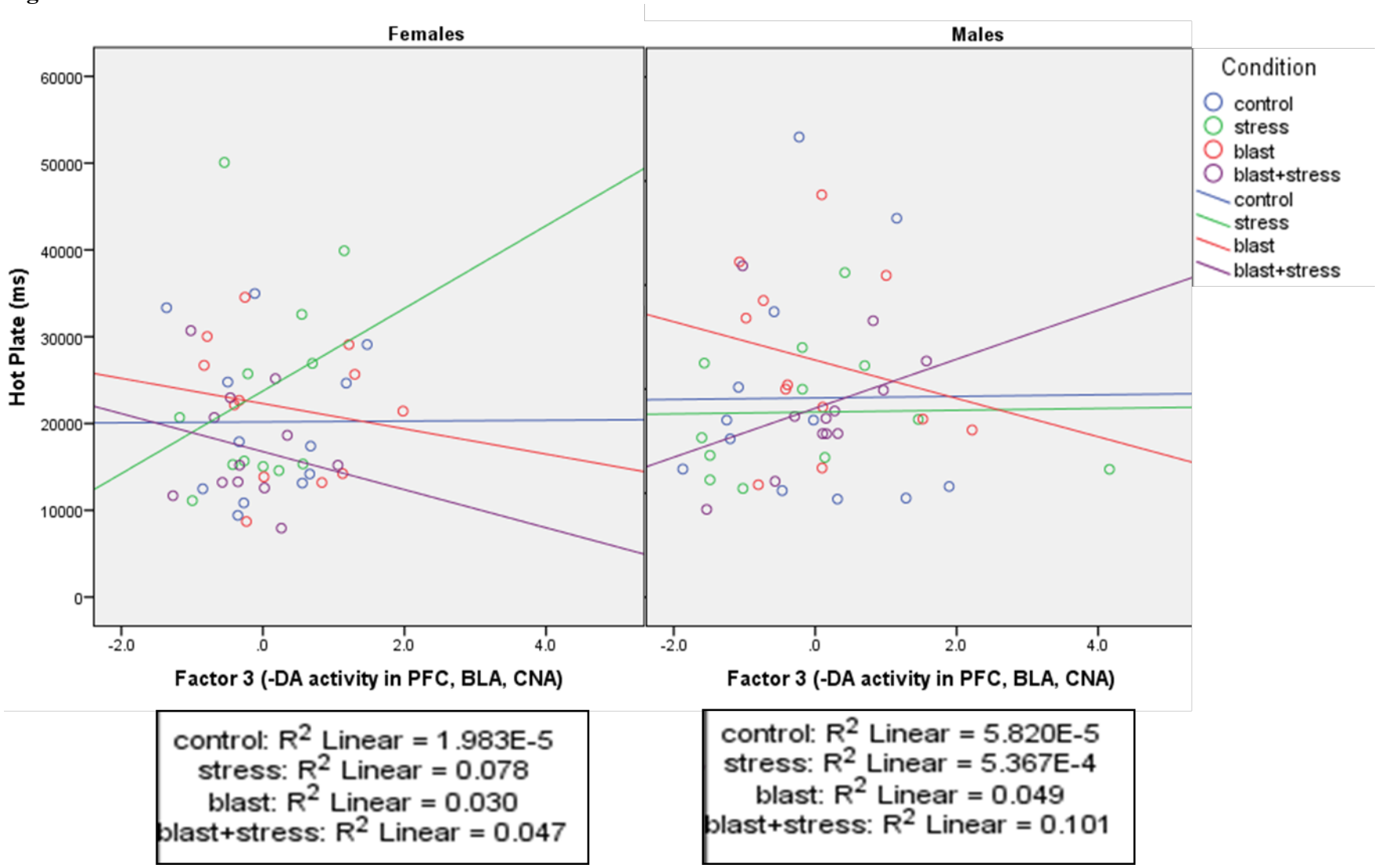


Figure 28 Factor 3 vs Hot Plate



Appendix B: Tables

Table 1. Parent Project Measures

Type of Variable	Variable Name
Behavioral	Passive Avoidance
	Acoustic Startle Response
	Hot Plate
	Open Field Activity
	Revised Neurobehavioral Severity Scale
	Rotarod
	Forced Swim Test
	Food & Water Consumption
Peripheral Measures	Corticosterone level
	ACTH level
	Prolactin level
	Immune Markers
Central Physiology	PFC DA activity
	PFC 5HT activity
	BLA DA activity
	BLA 5HT activity
	C NA DA activity
	C NA 5HT activity
	HIP DA activity
	HIP 5HT activity
	IC DA activity
	IC 5HT activity

Table Legend: PFC- prefrontal cortex; BLA- basolateral amgydala; CNA- central nucleus amygdala; HIP- hippocampus; IC- insula cortex; pg/uL- pica grams per micro liter

Table 2. Psychobiological Variables

Type of Variable	Variable Name	Units
Behavioral	Passive avoidance	Time (s)
	Acoustic Startle Response	startle units
	Hot Plate	Time (s)
Peripheral Physiology	Corticosterone Level	pg/uL
	ACTH Level	pg/uL
	Prolactin Level	pg/uL
Central Physiology	PFC dopamine activity	ratio
	PFC serotonin activity	ratio
	BLA dopamine activity	ratio
	BLA serotonin activity	ratio
	CNA dopamine activity	ratio
	CNA serotonin activity	ratio
	HIP dopamine activity	ratio
	HIP serotonin activity	ratio
	IC dopamine activity	ratio
	IC serotonin activity	ratio

Table Legend: PFC- prefrontal cortex; BLA- basolateral amgydala; CNA- central nucleus amygdala; HIP- hippocampus; IC- insula cortex; pg/uL- pica grams per micro liter

Table 3. Conceptual Multivariate Models

Type of Variable	Variable Name	Associated with Memory	Associated with Attention/Arousal	Associated with Pain
Behavioral	Passive Avoidance	**X**		
	Acoustic Startle Response		**X**	
	Hot Plate			**X**
Peripheral Physiology	Corticosterone level	**X**		**X**
	ACTH level			
	Prolactin level		**X**	
Central Physiology	PFC DA activity	**X**	**X**	
	PFC 5HT activity			
	BLA DA activity	**X**	**X**	
	BLA 5HT activity			**X**
	CNA DA activity	**X**	**X**	
	CNA 5HT activity			**X**
	HIP DA activity	**X**		
	HIP 5HT activity			**X**
	IC DA activity			
	IC 5HT activity			**X**

Table Legend: PFC- prefrontal cortex; DA- dopamine; 5HT- serotonin; BLA- basolateral amgydala; CNA- central nucleus amygdala; HIP- hippocampus; IC- insula cortex; pg/uL- pica grams per micro liter

Table 4. Significant correlations with Prolactin and ACTH

	Variable	Pearson's r	N	p
Prolactin	CORT	.34	74	.00
	HIP_5HT	.23	74	.05
	CNA_5HT	.24	74	.04
ACTH	HIP_5HT	.25	73	.03
	ASR PI_120	.25	73	.04

Table Legend: 5HT- serotonin; CNA- central nucleus amygdala; HIP- hippocampus; CORT- corticosterone; ASR_PI- startle response

Table 5. Examination of variables for normal distribution and univariate outliers

Variable	Skew/Kurtosis	Outliers	Transform
PFC_5HT	significantly	none > 3 SDs	Natural Log Log 10
PFC_DA	slight skew	none > 3 SDs	no
IC_5HT	significantly skewed	1 > 10 SDs from mean replaced with mean of group	Natural Log Log 10
IC_DA-	slightly skewed	none > 3 SDs	no
BLA_5HT	slightly skewed	1 > 5 SD from the mean replaced with the mean of the group	no
BLA_DA	significant kurtosis	none > 3 SDs	no
CNA_5HT	slight skew	none > 3 SDs	no
CNA_DA	slightly skewed	3 > 3 SDs; none > 4 SDs	no
HIP_5HT	skew improved after 1 outlier removed/very sig kurtosis	3 > 3 SDs; 2 > 4 SDs; 1 > 5 SDs removed 1 > 5 SDs replaced with the mean of the group	Natural Log Log 10
HIP_DA	significant kurtosis	2 > 5 SDs	Natural Log Log 10
HP_PI	very slightly skewed	1 > 3 SDs; none > 4 SDs	no
PA_Lat	significant negative skew and kurtosis	none > 3 SDs (not possible)	Natural Log Log 10 Dichotomized (cross/did not cross) converted to quartiles (< 100; 101 - 200; 201-299; 300)
ASR_PI	slight skew/non sig kurtosis	none > 3 SDs	no
CORT	slight skew/slight kurtosis	2 > 3 SDs; none > 4 SDs	no
PRO	slight skew/slight kurtosis	2 > 3 SDs; 1 > 5 SDs replaced with mean of the group	no
ACTH	none	5 > 3 SDs; 2 ~ 4 SDs	no

Table 6. Original Correlation Matrix

	Insula 5HT	HIP DA	PFC 5HT	HIP 5HT	PFC DA	Insula DA	BLA 5HT	BLA DA	C NA 5HT	C NA DA	Hot Plate	Acoustic Startle	CORT
Insula Serotonin	1.000	-.012	.529	.391	.023	.035	.316	.496	.038	.214	-.021	-.003	.001
Hippocampus Dopamine	-.012	1.000	-.001	.152	.218	.401	-.193	-.171	.139	-.277	-.070	.002	-.186
PFC Serotonin	.529	-.001	1.000	.493	.084	-.042	.383	.235	.090	.028	.057	.076	.103
Hippocampus Serotonin	.391	.152	.493	1.000	.041	.116	.252	.214	.190	.101	.081	.030	.019
PFC Dopamine	.023	.218	.084	.041	1.000	.245	-.002	.393	-.001	.281	.024	-.174	-.005
Insula Dopamine	.035	.401	-.042	.116	.245	1.000	.009	.169	.101	.125	-.015	.074	-.138
BLA Serotonin	.316	-.193	.383	.252	-.002	.009	1.000	.249	.248	.287	-.093	.047	.123
BLA Dopamine	.496	-.171	.235	.214	.393	.169	.249	1.000	-.089	.659	.089	-.044	.009
C NA Serotonin	.038	.139	.090	.190	-.001	.101	.248	-.089	1.000	-.010	-.017	-.005	.054
C NA Dopamine	.214	-.277	.028	.101	.281	.125	.287	.659	-.010	1.000	.021	-.090	.112
Hot Plate	-.021	-.070	.057	.081	.024	-.015	-.093	.089	-.017	.021	1.000	-.006	.246
Acoustic Startle	-.003	.002	.076	.030	-.174	.074	.047	-.044	-.005	-.090	-.006	1.000	.193
Corticosterone	.001	-.186	.103	.019	-.005	-.138	.123	.009	.054	.112	.246	.193	1.000

Table 7. Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy

Variables	KMO with PA	KMO without PA
IC_5HT	0.63	0.63
HIP_DA	0.50	0.52
PFC_5HT	0.63	0.64
HIP_5HT	0.74	0.74
PFC_DA	0.49	0.49
IC_DA	0.52	0.58
BLA_5HT	0.68	0.71
BLA_DA	0.59	0.59
CNA_5HT	0.56	0.54
CAN_DA	0.62	0.62
HP_POST_INJURY	0.42	0.37
PA_Test_Latency	0.40	
PI_120	0.34	0.37
CORT	0.49	0.49

Table 8. Communalities for all variables with passive avoidance in the model.

Insula Serotonin	.671
Hippocampus Dopamine	.767
PFC Serotonin	.715
Hippocampus Serotonin	.622
PFC Dopamine	.607
Insula Dopamine	.629
BLA Serotonin	.663
BLA Dopamine	.835
C NA Serotonin	.763
C NA Dopamine	.762
Hot plate	.658
Passive Avoidance	.534
Startle	.877
CORT	.677

Table 9. Reproduced Correlations from factor analysis model

	Insula 5HT	HIP DA	PFC 5HT	HIP 5HT	PFC DA	Insula DA	BLA 5HT	BLA DA	C NA 5HT	C NA DA	Hot Plate	Acoustic Startle	CORT
Insula Serotonin	.704[a]	-.047	.649	.540	.076	.014	.387	.492	-.055	.244	-.041	.024	-.067
Hippocampus Dopamine	-.047	.769[a]	.025	.224	.244	.541	-.254	-.189	.188	-.341	-.030	.021	-.284
PFC Serotonin	.649	.025	.730[a]	.636	-.036	-.053	.384	.282	.112	.039	.120	.074	.083
Hippocampus Serotonin	.540	.224	.636	.627[a]	.093	.124	.316	.230	.235	.007	.128	.021	.037
PFC Dopamine	.076	.244	-.036	.093	.608[a]	.425	-.045	.460	.031	.408	.168	-.276	-.067
Insula Dopamine	.014	.541	-.053	.124	.425	.705[a]	-.032	.211	.151	.149	-.100	.202	-.124
BLA Serotonin	.387	-.254	.384	.316	-.045	-.032	.684[a]	.301	.425	.367	-.194	.080	.163
BLA Dopamine	.492	-.189	.282	.230	.460	.211	.301	.835[a]	-.181	.722	.064	-.071	.044
C NA Serotonin	-.055	.188	.112	.235	.031	.151	.425	-.181	.814[a]	-.016	-.059	-.088	.148
C NA Dopamine	.244	-.341	.039	.007	.408	.149	.367	.722	-.016	.793[a]	.001	-.092	.148
Hot Plate	-.041	-.030	.120	.128	.168	-.100	-.194	.064	-.059	.001	.753[a]	-.049	.487
Acoustic Startle	.024	.021	.074	.021	-.276	.202	.080	-.071	-.088	-.092	-.049	.874[a]	.321
Corticosterone	-.067	-.284	.083	.037	-.067	-.124	.163	.044	.148	.148	.487	.321	.675[a]

Residuals were computed between observed and reproduced correlations. There were 35 (44.0%) nonredundant residuals with absolute values greater than 0.05.

Table 10. Summary of exploratory factor analysis results for psychobiological variables (N=96)

Psychobiological Variable	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Communalities
PFC Serotonin	.844						0.730
Insula Serotonin	.794						0.704
Hippocampus Serotonin	.750						0.627
Hippocampus Dopamine		.804					0.769
Insula Dopamine		.769					0.705
PFC Dopamine		.506	-.500				0.608
CNA Dopamine			-.881				0.793
BLA Dopamine			-.858				0.835
Hot plate				.847			0.753
Corticosterone				.665			0.675
CN A Serotonin					.869		0.814
BLA Serotonin	.442				.608		0.684
Acoustic startle						.912	0.874
Eigenvalues	2.294	1.693	2.204	1.257	1.321	1.212	
% of variance	17.64745	13.02039	16.95162	9.671923	10.15835	9.319866	
% covariance	22.98755	16.96034	22.08116	12.59864	13.23226	12.14005	

˙Note: Blanks indicate factor loadings < .4. Factors are ordered based on initial extraction; all values presented here represent estimates after rotation was conducted. Therefore, eigenvalues do not appear in descending order.

Table 11. Summary of cannonical discriminant functions

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	0.731	46.8	46.8	.650
2	0.376	24.1	70.9	.523
3	0.198	12.7	83.6	.406
4	0.131	8.4	92.0	.340
5	0.082	5.2	97.2	.275
6	0.026	1.6	98.8	.158
7	0.018	1.2	100.0	.133

Table 12. Pooled within group correlations on each discriminant function

	Function						
	1	**2**	**3**	**4**	**5**	**6**	**7**
BLA_5HT	**.524***	.026	-.259	.316	-.047	.172	.236
HIP_5HT	**.510***	-.160	-.320	.318	.274	-.254	-.267
CORT	.192	**.451***	-.130	.304	-.260	.255	-.058
ASR (PI_120)	-.014	-.182	**-.528***	-.103	.360	.385	.097
PFC_DA	.323	.034	.280	**-.442***	-.284	-.176	.315
HP_POST_INJURY	-.065	.350	-.100	-.109	**.445***	-.204	-.065
Memory (PA_TRI)	.022	**-.488**	.430	.254	.095	.560*	.194
BLA_DA	.497	-.266	.249	-.131	.116	-.543*	.213
IC_DA	**.007**	.128	.169	.268	**.395**	-.284	.606*
IC_5HT	**.518**	-.021	.310	.090	.412	-.111	-.521*
PFC_5HT	.274	-.089	-.238	**.314**	-.046	-.349	-.496*

Table 13. Summary of multiple discriminant analysis split by sex

Sex	Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation	Function	Wilks' Lambd	Chi-square	df	Sig.
	1	0.485	52.9	52.9	.572	1 through 3	.458	30.850	33	.575
Females	2	0.313	34.1	86.9	.488	2 through 3	.680	15.221	20	.764
	3	0.12	13.1	100.0	.327	3	.893	4.476	9	.877
	1	0.675	60.0	60.0	.635	1 through 3	.399	36.309	33	.317
Males	2	0.282	25.1	85.1	.469	2 through 3	.668	15.941	20	.720
	3	0.168	14.9	100.0	.379	3	.856	6.131	9	.727

Table 14. Pooled within-cell correlations between Memory dependent variables

	PA_TRI	CORT	PFC_DA	BLA_DA	CAN_DA
PA_TRI					
CORT	-.011				
PFC_DA	.082	-.037			
BLA_DA	-.009	.004	.326		
CAN_DA	.095	.136	.242	**.659**	
HIP_DA	.179	-.188	.250	-.147	-.275

Table 15. Correlation coefficients for internal analyses

Sex	Condition	ACTH & IC dopamine	ACTH & BLA dopamine	ACTH & HIP dopamine	ASR & PFC dopamine
Females	control	.423	-.025	.196	.198
	stress	.410	.143	-.127	-.251
	blast	.427	.281	-.319	.609*
	blast + stress	.088	.510	-.399	-.004
Males	Control	-.020	.348	-.541	-.492
	Stress	.487	-.115	.663*	-.014
	Blast	.664*	.632*	.239	-.551
	blast + stress	.159	.240	.302	-.216

* correlation significant, $p < .05$

Appendix C: Assessment for multivariate outliers

The dataset (including 14 variables; *i.e.,* excluding ACTH and PRO) was screened for mulitivariate outliers using Mahalanobis distance, derived using SPSS Linear Regression, and evaluated using χ^2 distribution, $p < .001$, $df = 14$ (the number of variables entered in regression), $\chi^2(14) = 36.123$. Mahalanobis distance represents the distance of a case from the centroid of the remaining cases, or the point where means of all variables intersect (Tabachnick & Fidell, 2007). Cases that fall beyond a certain distance (determined using the χ^2 distribution) may be a multivariate outlier and may need to be removed from the analysis. Based on the criteria listed above, five multivariate outliers were identified and the Regression model revealed five variables significantly predicted outlier status. One case had two multivariate outliers, but only the case for one of the variables was > 4 SDs outside the mean; this case was replaced by the mean ratio value of the group and then the natural log transformation was re-calculated. The regression was then run again to determine the number of remaining outliers. There were three remaining outliers for five variables. One case had a multivariate outlier > 4 SDs from the mean; this case was replaced by the mean ratio value of the group and then the natural log transformation was recalculated. The regression was then run again to determine the number of remaining outliers. There were three outliers remaining for four variables. All cases were within 4 SDs; therefore, they were retained. No further adjustments were needed.

Appendix D: Further examination of findings

Based on different patterns for males and females that were present in *all analyses where effects of sex were tested*, separate exploratory factor analyses (EFA) were conducted on male and female datasets. EFA procedures (as described above in the EFA section) were followed: including examining correlations among variables; determining adequacy of sample size; extracting factors; and performing orthogonal rotation.

The results for females revealed that it was possible to derive interpretable, underlying factors based on the variables entered and sample size (N = 48). In contrast, the results for males revealed that the EFA model was not sufficient to detect similar interpretable factors based on sample size (N = 48). This differential finding for males and females was puzzling because the experiment included identical numbers of male and female rats exposed to the same experimental procedures. To try to figure out why this happened, the correlations among variables within each sex were examined because EFA models are based on the strength of correlations among variables.

There were significant bivariate correlations within each sex (16 for females and 10 for males), but males and females only shared five significant bivariate correlations. The large number of significant correlations that differed between males and females was surprising. Three of the correlations that were significant for females but not males involved relationships between peripheral and central biochemistry, and one of the correlations that was significant for males but not females involved the relationship between behavior and central biochemistry. To try to understand if these sex differences in relationships might help to explain why the hypothesized effects of stress did not occur, correlation coefficients were compared in search of patterns that differentiated

stress conditions for males and females (see Table 15). Although strengths of correlations differed between groups, no clear patterns emerged. Therefore, another approach was attempted.

This other approach examined how males and females might differ with regard to the first three factors derived from the EFA (*i.e.,* serotonin activity among the PFC, IC, HIP, BLA; dopamine activity among the PFC, IC, HIP; decrement of dopamine activity among the PFC, CNA, BLA; see EFA section of text for details) and how these factors related to two behaviors based on stress condition (*i.e.,* hot plate, ASR). The data were split by sex and bivariate scatterplots were created. A linear "best fit line" was calculated for each sub-group (see Figures 23-28).

Visual inspection of these figures reveals apparent differences for stress conditions for each sex as well as differences between males and females. However, no clear patterns emerged.

Summary. Because males and females differed with regard to the psychobiological variables under study, the data were examined between stress conditions in search of patterns that might distinguish between the male and female responses. No clear patterns emerged.

Appendix E. MANOVA Summary Tables

Table E1. 4 x 2 MANOVA summary table for 6 Factors

	Test Statistic	Value	F	Hypothesis df	Error df	Sig.	Observed Power
Sex	Pillai's Trace	.257	4.776	6	83.00	.000	.986
	Wilks' Lambda	.743	4.776	6	83.00	.000	.986
	Hotelling's Trace	.345	4.776	6	83.00	.000	.986
	Roy's Largest Root	.345	4.776	6	83.00	.000	.986
Condition	Pillai's Trace	.164	.818	18	255.00	.678	.594
	Wilks' Lambda	.844	.805	18	235.24	.694	.549
	Hotelling's Trace	.175	.792	18	245.00	.709	.574
	Roy's Largest Root	.085	1.201	6	85.00	.314	.449
Sex * Condition	Pillai's Trace	.113	.555	18	255.00	.929	.397
	Wilks' Lambda	.890	.547	18	235.24	.933	.365
	Hotelling's Trace	.119	.541	18	245.00	.936	.386
	Roy's Largest Root	.064	0.909	6	85.00	.493	.341

Table E2. Between-subjects effects for 4 x 2 MANOVA for 6 Factors

	Factor	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta²	Observed Power
Sex	Factor 1	15.062	1	15.062	17.529	.000	.166	.985
	Factor 2	.043	1	.043	.041	.841	.000	.055
	Factor 3	12.495	1	12.495	13.515	.000	.133	.953
	Factor 4	.055	1	.055	.055	.816	.001	.056
	Factor 5	.931	1	.931	.922	.339	.010	.158
	Factor 6	.032	1	.032	.032	.859	.000	.054
Condition	Factor 1	2.791	3	.930	1.083	.361	.036	.284
	Factor 2	.981	3	.327	.311	.818	.010	.108
	Factor 3	.874	3	.291	.315	.814	.011	.109
	Factor 4	2.922	3	.974	.973	.409	.032	.257
	Factor 5	2.030	3	.677	.671	.572	.022	.186
	Factor 6	5.193	3	1.731	1.706	.172	.055	.432
Sex * Condition	Factor 1	1.531	3	.510	.594	.621	.020	.169
	Factor 2	1.355	3	.452	.429	.733	.014	.133
	Factor 3	.274	3	.091	.099	.961	.003	.067
	Factor 4	3.954	3	1.318	1.317	.274	.043	.340
	Factor 5	3.233	3	1.078	1.068	.367	.035	.280
	Factor 6	.495	3	.165	.163	.921	.006	.079
Error	Factor 1	75.616	88	.859				
	Factor 2	92.621	88	1.053				
	Factor 3	81.357	88	.925				
	Factor 4	88.069	88	1.001				
	Factor 5	88.806	88	1.009				
	Factor 6	89.280	88	1.015				
Total	Factor 1	95.000	95					
	Factor 2	95.000	95					
	Factor 3	95.000	95					
	Factor 4	95.000	95					
	Factor 5	95.000	95					
	Factor 6	95.000	95					

Table E3. 4 x 2 MANOVA summary table for 5 Factors

	Test Statistic	Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Observed Power
Sex	Pillai's Trace	.257	5.8	5	84.00	.000	.257	.991
	Wilks' Lambda	.743	5.8	5	84.00	.000	.257	.991
	Hotelling's Trace	.345	5.8	5	84.00	.000	.257	.991
	Roy's Largest Root	.345	5.8	5	84.00	.000	.257	.991
Condition	Pillai's Trace	.114	.682	15	258.00	.802	.038	.447
	Wilks' Lambda	.890	.671	15	232.29	.812	.038	.400
	Hotelling's Trace	.120	.660	15	248.00	.822	.038	.432
	Roy's Largest Root	.060	1.029	5	86.00	.406	.056	.351
Sex * Condition	Pillai's Trace	.110	.653	15	258.00	.829	.037	.427
	Wilks' Lambda	.893	.645	15	232.29	.835	.037	.384
	Hotelling's Trace	.116	.639	15	248.00	.842	.037	.417
	Roy's Largest Root	.064	1.1	5	86.00	.366	.060	.374

Table E4. Between-subjects effects for 4 x 2 MANOVA for 6 Factors

	Factor	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta²	Observed Power
Sex	Factor 1	15.062	1	15.062	17.529	.000	.166	.985
	Factor 2	.043	1	.043	.041	.841	.000	.055
	Factor 3	12.495	1	12.495	13.515	.000	.133	.953
	Factor 4	.055	1	.055	.055	.816	.001	.056
	Factor 5	.931	1	.931	.922	.339	.010	.158
Condition	Factor 1	2.791	3	.930	1.083	.361	.036	.284
	Factor 2	.981	3	.327	.311	.818	.010	.108
	Factor 3	.874	3	.291	.315	.814	.011	.109
	Factor 4	2.922	3	.974	.973	.409	.032	.257
	Factor 5	2.030	3	.677	.671	.572	.022	.186
Sex * Condition	Factor 1	1.531	3	.510	.594	.621	.020	.169
	Factor 2	1.355	3	.452	.429	.733	.014	.133
	Factor 3	.274	3	.091	.099	.961	.003	.067
	Factor 4	3.954	3	1.318	1.317	.274	.043	.340
	Factor 5	3.233	3	1.078	1.068	.367	.035	.280
Error	Factor 1	75.616	88	.859				
	Factor 2	92.621	88	1.053				
	Factor 3	81.357	88	.925				
	Factor 4	88.069	88	1.001				
	Factor 5	88.806	88	1.009				
Total	Factor 1	95.000	95					
	Factor 2	95.000	95					
	Factor 3	95.000	95					
	Factor 4	95.000	95					
	Factor 5	95.000	95					

Table E5. Summary table for MANOVAs split by females and males

Sex	Statistic	Value	F	Hypothesis df	Error df	Sig.	Partial Eta²	Observed Power
Females	Pillai's Trace	.395	1.035	18	123.00	.427	.132	.696
	Wilks' Lambda	.651	1.008	18	110.79	.456	.133	.638
	Hotelling's Trace	.468	.980	18	113.00	.487	.135	.659
	Roy's Largest Root	.262	1.794	6	41.00	.124	.208	.605
Males	Pillai's Trace	.281	.707	18	123.00	.799	.094	.485
	Wilks' Lambda	.738	.697	18	110.79	.808	.096	.442
	Hotelling's Trace	.328	.686	18	113.00	.818	.099	.466
	Roy's Largest Root	.204	1.397	6	41.00	.239	.170	.483

Table E6. Between-subjects effects for MANOVA split by females and males

		Factor	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta²	Observed Power
Females	Condition	Factor 1	.319	3	.106	.204	.893	.014	.085
		Factor 2	1.753	3	.584	.943	.428	.060	.241
		Factor 3	.314	3	.105	.227	.877	.015	.089
		Factor 4	2.316	3	.772	.683	.567	.044	.182
		Factor 5	1.770	3	.590	1.240	.307	.078	.309
		Factor 6	2.592	3	.864	.953	.423	.061	.243
	Error	Factor 1	22.944	44	.521				
		Factor 2	27.271	44	.620				
		Factor 3	20.230	44	.460				
		Factor 4	49.745	44	1.131				
		Factor 5	20.938	44	.476				
		Factor 6	39.884	44	.906				
	Total	Factor 1	23.264	47					
		Factor 2	29.024	47					
		Factor 3	20.543	47					
		Factor 4	52.061	47					
		Factor 5	22.709	47					
		Factor 6	42.476	47					
Males	Condition	Factor 1	4.003	3	1.334	1.115	.353	.071	.280
		Factor 2	.583	3	.194	.131	.941	.009	.072
		Factor 3	.834	3	.278	.200	.896	.013	.084
		Factor 4	4.560	3	1.520	1.745	.172	.106	.424
		Factor 5	3.493	3	1.164	.755	.525	.049	.198
		Factor 6	3.096	3	1.032	.919	.439	.059	.235
	Error	Factor 1	52.672	44	1.197				
		Factor 2	65.350	44	1.485				
		Factor 3	61.128	44	1.389				
		Factor 4	38.324	44	.871				
		Factor 5	67.868	44	1.542				
		Factor 6	49.396	44	1.123				
	Total	Factor 1	56.674	47					
		Factor 2	65.933	47					
		Factor 3	61.962	47					
		Factor 4	42.885	47					
		Factor 5	71.361	47					
		Factor 6	52.492	47					

Table E7. 4 x 2 MANOVA for variables associated with memory

	Test Statistic	Value	F	Hypothesis df	Error df	Sig.	Partial Eta²	Observed Power
Sex	Pillai's Trace	.216	3.803	6.000	83.000	.002	.216	.953
	Wilks' Lambda	.784	3.803	6.000	83.000	.002	.216	.953
	Hotelling's Trace	.275	3.803	6.000	83.000	.002	.216	.953
	Roy's Largest Root	.275	3.803	6.000	83.000	.002	.216	.953
Condition	Pillai's Trace	.110	.540	18.000	255.000	.937	.037	.386
	Wilks' Lambda	.893	.534	18.000	235.245	.940	.037	.355
	Hotelling's Trace	.117	.529	18.000	245.000	.943	.037	.376
	Roy's Largest Root	.077	1.084	6.000	85.000	.379	.071	.406
Sex * Condition	Pillai's Trace	.285	1.484	18.000	255.000	.095	.095	.902
	Wilks' Lambda	.737	1.491	18.000	235.245	.094	.097	.878
	Hotelling's Trace	.329	1.494	18.000	245.000	.092	.099	.903
	Roy's Largest Root	.215	3.04	6.000	85.000	.010	.177	.891

Table E8. Between-subjects effects for 4 x 2 MANOVA for memory

	Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta²	Observed Power
Sex	Pass Avoid	0.51	1	0.51	0.33	.565	.004	.088
	CORT	11106.10	1	11106.10	1.17	.283	.013	.188
	PFC DA Activity	1.89	1	1.89	7.56	.007	.079	.776
	BLA DA Activity	0.45	1	0.45	16.98	.000	.162	.983
	C.NA DA Activity	0.01	1	0.01	3.38	.069	.037	.444
	HIP DA Activity	0.25	1	0.25	0.69	.410	.008	.130
Condition	Pass Avoid	3.11	3	1.04	0.68	.568	.023	.188
	CORT	28672.05	3	9557.35	1.00	.395	.033	.265
	PFC DA Activity	0.46	3	0.15	0.62	.605	.021	.174
	BLA DA Activity	0.03	3	0.01	0.35	.789	.012	.116
	C.NA DA Activity	0.00	3	0.00	0.36	.779	.012	.119
	HIP DA Activity	0.23	3	0.08	0.20	.893	.007	.087
Sex * Condition	Pass Avoid	15.86	3	5.29	3.45	.020	.105	.756
	CORT	65704.40	3	21901.47	2.30	.083	.073	.562
	PFC DA Activity	0.44	3	0.15	0.58	.630	.019	.166
	BLA DA Activity	0.06	3	0.02	0.75	.523	.025	.206
	C.NA DA Activity	0.00	3	0.00	0.30	.829	.010	.105
	HIP DA Activity	0.51	3	0.17	0.46	.712	.015	.139
Error	Pass Avoid	134.92	88	1.53				
	CORT	837434.68	88	9516.30				
	PFC DA Activity	22.03	88	0.25				
	BLA DA Activity	2.31	88	0.03				
	C.NA DA Activity	0.31	88	0.00				
	HIP DA Activity	32.57	88	0.37				
Total	Pass Avoid	154.41	95					
	CORT	942917.24	95					
	PFC DA Activity	24.82	95					
	BLA DA Activity	2.84	95					
	C.NA DA Activity	.33	95					
	HIP DA Activity	33.56	95					

Table E9. 4 x 2 MANOVA for variables associated with attention

	Test Statistic	Value	F	Hypothesis df	Error df	Sig.	Partial Eta²	Observed Power
Sex	Pillai's Trace	.191	5.009	4.000	85.000	.001	.191	.954
	Wilks' Lambda	.809	5.009	4.000	85.000	.001	.191	.954
	Hotelling's Trace	.236	5.009	4.000	85.000	.001	.191	.954
	Roy's Largest Root	.236	5.009	4.000	85.000	.001	.191	.954
Condition	Pillai's Trace	.105	.788	12.000	261.000	.663	.035	.458
	Wilks' Lambda	.897	.785	12.000	225.180	.666	.035	.397
	Hotelling's Trace	.112	.781	12.000	251.000	.670	.036	.453
	Roy's Largest Root	.086	1.863	4.000	87.000	.124	.079	.544
Sex * Condition	Pillai's Trace	.095	.714	12.000	261.000	.738	.032	.414
	Wilks' Lambda	.907	.705	12.000	225.180	.746	.032	.355
	Hotelling's Trace	.100	.697	12.000	251.000	.754	.032	.403
	Roy's Largest Root	.066	1.427	4.000	87.000	.232	.062	.427

Table E10. Between-subjects effects for 4 x 2 MANOVA for attention

	Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta²	Observed Power
Sex	Startle	394.86	1	394.86	0.00	.968	.000	.050
	PFC DA Activity	1.89	1	1.89	7.56	.007	.079	.776
	BLA DA Activity	0.45	1	0.45	16.98	.000	.162	.983
	C.NA DA Activity	0.01	1	0.01	3.38	.069	.037	.444
Condition	Startle	1625033.23	3	541677.74	2.23	.091	.071	.547
	PFC DA Activity	0.46	3	0.15	0.62	.605	.021	.174
	BLA DA Activity	0.03	3	0.01	0.35	.789	.012	.116
	C.NA DA Activity	0.00	3	0.00	0.36	.779	.012	.119
Sex * Condition	Startle	165085.79	3	55028.60	0.23	.878	.008	.091
	PFC DA Activity	0.44	3	0.15	0.58	.630	.019	.166
	BLA DA Activity	0.06	3	0.02	0.75	.523	.025	.206
	C.NA DA Activity	0.00	3	0.00	0.30	.829	.010	.105
Error	Startle	21395131.19	88	243126.49				
	PFC DA Activity	22.03	88	0.25				
	BLA DA Activity	2.31	88	0.03				
	C.NA DA Activity	0.31	88	0.00				
Total	Startle	23185645.06	95					
	PFC DA Activity	24.82	95					
	BLA DA Activity	2.84	95					
	C.NA DA Activity	.33	95					

Table E11. 4 x 2 MANOVA for variables associated with pain

	Test Statistic	Value	F	Hypothesis df	Error df	Sig.	Partial Eta²	Observed Power
Sex	Pillai's Trace	.315	6.348	6.000	83.000	.000	.315	.998
	Wilks' Lambda	.685	6.348	6.000	83.000	.000	.315	.998
	Hotelling's Trace	.459	6.348	6.000	83.000	.000	.315	.998
	Roy's Largest Root	.459	6.348	6.000	83.000	.000	.315	.998
Condition	Pillai's Trace	.190	.959	18.000	255.000	.508	.063	.685
	Wilks' Lambda	.821	.943	18.000	235.245	.527	.064	.637
	Hotelling's Trace	.204	.927	18.000	245.000	.547	.064	.664
	Roy's Largest Root	.093	1.314	6.000	85.000	.260	.085	.489
Sex * Condition	Pillai's Trace	.163	.812	18.000	255.000	.685	.054	.590
	Wilks' Lambda	.844	.809	18.000	235.245	.689	.055	.551
	Hotelling's Trace	.178	.806	18.000	245.000	.693	.056	.584
	Roy's Largest Root	.112	1.592	6.000	85.000	.159	.101	.583

Table E12. Between-subjects effects for 4 x 2 MANOVA for pain

	Variables	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta²	Power
Sex	BLA 5HT Activity	0.51	1	0.51	15.15	.000	.147	.971
	C.NA 5HT Activity	0.01	1	0.01	0.05	.820	.001	.056
	Hot Plate	36299760.67	1	36299760.67	0.48	.492	.005	.105
	CORT	11106.10	1	11106.10	1.17	.283	.013	.188
	IC 5HT Activity	2.75	1	2.75	17.16	.000	.163	.984
	HIP 5HT Activity	0.51	1	0.51	14.69	.000	.143	.966
Condition	BLA 5HT Activity	0.07	3	0.02	0.68	.568	.023	.188
	C.NA 5HT Activity	0.17	3	0.06	0.39	.763	.013	.123
	Hot Plate	251427386.71	3	83809128.90	1.10	.355	.036	.287
	CORT	28672.05	3	9557.35	1.00	.395	.033	.265
	IC 5HT Activity	0.49	3	0.16	1.01	.390	.033	.267
	HIP 5HT Activity	0.16	3	0.05	1.55	.208	.050	.395
Sex * Condition	BLA 5HT Activity	0.10	3	0.03	0.99	.402	.033	.261
	C.NA 5HT Activity	0.27	3	0.09	0.60	.618	.020	.170
	Hot Plate	182581691.08	3	60860563.69	0.80	.499	.026	.216
	CORT	65704.40	3	21901.47	2.30	.083	.073	.562
	IC 5HT Activity	0.08	3	0.03	0.16	.921	.006	.079
	HIP 5HT Activity	0.07	3	0.02	0.67	.573	.022	.186
Error	BLA 5HT Activity	2.97	88	0.03				
	C.NA 5HT Activity	13.02	88	0.15				
	Hot Plate	6720177913.5	88	76365658.11				
	CORT	837434.68	88	9516.30				
	IC 5HT Activity	14.11	88	0.16				
	HIP 5HT Activity	3.08	88	0.03				
Total	BLA 5HT Activity	3.65	95					
	C.NA 5HT Activity	13.74	95					
	Hot Plate	7190486751.9	95					
	CORT	942917.24	95					
	IC 5HT Activity	17.42	95					
	HIP 5HT Activity	3.82	95					